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# ANNUAL RESEARCH PROGRESS REPORT

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) During Fiscal Year 1977 progress was attained at the Letterman Army Institute of Research in the following research areas: basic and applied research in skin diseases of military importance; the effects of hemorrhagic shock on the heart and brain; basic nutritional biochemistry; basic biochemical processes of metabolism; basic and applied nutrition; clinical nutrition; the metabolism of normal man and as altered by disease; the evaluation of insect repellent; serodiagnosis of leishmaniasis; the determination of exposure thresholds of coherent (see reverse)		

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radiation producing damage to the eye and skin; basic and applied studies on blood and blood products storage; work performance on man and military dogs; and research computer science. The progress made in this fiscal year is described in the reports of the work units presented.

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## FOREWORD

The research conducted at the Letterman Army Institute of Research, Presidio of San Francisco, California, was accomplished in Fiscal Year 1977 under the following Department of the Army projects:

3A161101A91C - In House Laboratory Independent Research

3M161102BS02 - Basic Mechanisms of Recovery from Injury

3M762772A810 - Military Skin Disease

3M762772A811 - Military Nutrition and Food Hygiene

3M762772A812 - Military Research Animal Resources

3E762772A813 - Health Effects of Military Lasers

3S762772A814 - Military Trauma and Resuscitation

Projects are subdivided into work units and studies, as appropriate, to accomplish project objectives.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animals Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACT <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DDB'S INST <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM <sup>a</sup>
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11. NO. / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	61101A	3A161101A91C		00	040		
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C. OTHER	NONE						
12. TITLE (Precede with Security Classification Code)							
(U) The Molecular Basis of Vitamin A Activity (06)							
13. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>a</sup>							
002300 Biochemistry							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
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21. RESPONSIBLE ODD ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				NAME <sup>a</sup> Letterman Army Institute of Research ADDRESS <sup>a</sup> Biochemistry Division Department of Nutrition Presidio of San Francisco, CA 94129 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME <sup>a</sup> Sauberlich, H.E., DAC TELEPHONE 415-561-4323 SOCIAL SECURITY ACCOUNT NUMBER			
RESPONSIBLE INDIVIDUAL NAME: Canham, J.E., COL, MC TELEPHONE 415-561-3600				ASSOCIATE INVESTIGATORS NAME: Bashor, M.M., DAC NAME: POC:DA			
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24. KEYWORDS (Precede EACH with Security Classification Code)							
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25. TECHNICAL OBJECTIVE <sup>a</sup> 26. APPROACH 27. PROGRESS (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
<p>23. (U) The function of vitamin A, aside from its participation in vision, remains unknown; yet the effects of a deficiency of vitamin A are marked and diverse. Of military interest is its apparent participation in resistance to infection and stress and its requirements in wound healing. Vitamin A is also responsible for normal keratinization in epithelial tissues. In order to provide a better rationale for the use of vitamin A and its requirements, investigations will be conducted on vitamin A functions at the molecular level. The studies will be designed to determine the specific tissue functions of vitamin A and its active form.</p> <p>24. (U) Studies will be conducted on the involvement of vitamin A on sulfur amino acid metabolism and sulfation, on membrane carrier lipids, on erythrocytes and membrane functions, on wound healing and infection, on amino acid incorporation and enzyme synthesis, and on DNA and RNA metabolism. Studies will use tissue culture techniques, cellular blocking agents, labeled substrates, electron microscopy, and other methods.</p> <p>25. (U) 76 10 - 77 09 Column chromatographic techniques are being developed and employed to (1) isolate and purify human retinol binding protein (RBP) for use in studies of wound healing, and (2) prepare immunochemical assays for RBP to provide nutritional and toxicological evaluations. Cell culture systems are being investigated as useful alternatives to the more conventional and expensive whole-animal studies. Results suggest that vitamin deficiencies can be induced in animal cell cultures even though undefined animal sera must be included in the growth medium.</p>							

# ABSTRACT

PROJECT NO.	3A161101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	040	The Molecular Basis of Vitamin A Activity

The following investigation has been conducted under this work unit:

STUDY NO.	2	Development of a microfluorometric assay for vitamin A
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At the present time the most commonly employed assay for vitamin A (retinol) is based on a colorimetric procedure whereby the vitamin is extracted into an organic solvent and reacted with trifluoroacetic acid in the presence of chloroform and acetic anhydride to produce a blue color, the intensity of which is proportional to the concentration of the vitamin A. Problems associated with this procedure, relevant to this project, are the large sample required and the use of the highly volatile, inflammable, and expensive organic solvents. A direct microfluorometric procedure was recently described in the literature. The procedure relies on the enhancement of the fluorescence of retinol when it is bound to its serum transport protein, retinol binding protein (RBP). We have compared fluorescence determinations made on a number of plasma samples with vitamin A determinations made by a colorimetric method as well as with immunological determinations of plasma RBP. Such studies indicate that a good correlation exists between the two methods. The direct microfluorometric method does not require extensive sample preparation or extraction and less than 50  $\mu$ l of sample are required. It is necessary to run a fluorescence excitation spectrum of each sample to insure that samples containing interfering substances can be identified. The peak of fluorescence by tyrosine and tryptophan of the plasma proteins at 292 nm excitation does not interfere with the peak of retinol-RBP fluorescence at 335-340 nm. Preliminary results of ion-exchange and gel filtration fractionations of human plasma indicate that there may exist in plasma a protein, distinct from RBP which nevertheless has a fluorescence excitation spectrum similar to RBP.



## BODY OF REPORT

WORK UNIT NO. 040

The Molecular Basis of Vitamin A Activity

STUDY NO. 2

Development of a microfluorometric assay for vitamin A

### PROBLEM

Vitamin A is essential for maintaining vision, skeletal development, reproductive capacity, and normal differentiation of many epithelial tissues (skin, trachea, etc.). With the exception of the role of vitamin A as the light-sensitive prosthetic group of the visual pigments, the mechanism(s) of action is (are) not yet known. This lack of basic knowledge has undoubtedly hindered the development of new therapies involving vitamin A. Recent progress in the vitamin A field has led to the use of derivatives of the vitamin for the effective treatment of cancer in tissues of epithelial origin. The importance of vitamin A nutrition in wound healing, resistance to infection, coping with stress, and vision makes this an area of research obviously important to the military forces. The development and evaluation of a rapid sensitive assay for vitamin A are necessary for both practical applied studies as well as for experimental studies of the mechanism(s) of action of vitamin A.

### RESULTS AND DISCUSSION OF RESULTS

At present the methods of evaluating vitamin A status are limited to immunochemical detection of retinol binding protein (RBP) or extraction of retinol from plasma into an organic solvent followed by spectrophotometric, fluorometric, or colorimetric determinations. Some of the fluorometric methods published provide for the interference due to a fluorescent carotene (phytofluene) but ignore the interference due to quenching by the colored carotenes. We have shown that beta-carotene can quench fluorescence by more than 90% depending upon its concentration. Other procedures published in the literature ignore the effects of both fluorescent contaminants as well as compounds which quench the fluorescence of retinol. A few procedures recommend the separation of retinol from contaminants by column chromatography. Of the fluorescent methods tried in this laboratory, two seem to offer the best results. One method involves the extraction of retinol, followed by chromatography on silicic acid. This sequence is rather laborious but does separate retinol from both phytofluene and beta-carotene. While this method is reproducible on a sample-to-sample basis, on any given day, the day-to-day variation in recovery of retinol varies between 85 to 98% (although it is reproducible on a given day). This requires that standards must be run with great care each day. A second fluorometric method which is currently being evaluated is a direct fluorometric method whereby the sample of plasma is diluted in buffer and

the fluorescence is directly measured. This is possible since the fluorescence yield of retinol is increased when it is bound to its transport protein RBP. The fluorescence spectrum of RBP, as well as diluted plasma containing RBP, has a distinctive pattern, with peaks at 292 nm (due to tyrosine and tryptophan) and 335-340 nm due to retinol. We have found a linear relationship between plasma concentration and fluorescence at 335 nm over a wide range of concentrations. Routinely we use 25  $\mu$ l of plasma diluted 100-fold with normal saline. Such a dilution should eliminate interference due to carotenes. There still exists in some plasma samples a substance or substances which alter the shape of the fluorescence spectrum to such a degree that an estimation of the retinol concentration is not possible by measuring the fluorescence at 335 nm. It is therefore necessary to run a spectrum of each sample to insure that a "normal" profile is being measured. It has long been known that retinol is light sensitive and it is this characteristic that has been used as the basis of a spectrophotometric assay for retinol as follows. The vitamin is extracted into an appropriate organic solvent, evaporated to dryness, redissolved in a small volume of solvent and the absorbance read before and after irradiation with ultraviolet light. The difference in readings is divided by the extinction coefficient for retinol to obtain the concentration of retinol in the extract. It has been shown, however, that there are other substances present in such extracts which also absorb at 325 nm, and which are also destroyed by ultraviolet irradiation. Experiments in this and other laboratories have shown that retinol bound to RBP is protected to a certain extent against ultraviolet destruction. Under specified conditions it may require 10 h of illumination to destroy the retinol in plasma, but only 1 to 2 h to accomplish this in a hexane extract. Although irradiation of plasma for 10 h eliminates the fluorescent peak at 335 nm, the background fluorescence nevertheless remains quite high (and constant) between approximately 330-380 nm. This suggests that it may be more appropriate to measure the fluorescence intensity of the samples before and after irradiation and relate the difference in these two readings to the content of retinol. Efforts should also be made to identify the source of the background fluorescence. Chromatographic isolation of RBP for the purposes of setting up radial immunodiffusion assays for the protein and to obtain purified RBP for studies on wound healing suggest that at least one other protein is present in human plasma with a fluorescence excitation spectrum similar to RBP. Experiments are underway to isolate and identify this protein and to determine what interference, if any, it may impart to the microfluorometric procedure.

#### CONCLUSION

A direct microfluorometric assay for retinol appears suitable for plasma and serum vitamin A determinations.



### RECOMMENDATIONS

1. Work should be continued with microfluorometric retinol assay to determine whether or not an interfering protein exists in human plasma.
2. Work should be continued on RBP purification to support recommendations 1 and 3 and to prepare antisera and hence a radial immunodiffusion assay for plasma RBP.
3. Tissue/organ cultures studies should be initiated in support of work to be done on the metabolism of, and the effects of, RBP on wound healing.

### PUBLICATIONS

1. PLOPPER, C.G., D.L. WALLACE, T.J. BUCCI, and H.F. SAUBERLICH. Autoradiographic localization of vitamin A in the kidney of rats. Proc Soc Exptl Biol Med 155:124, 1977
2. BASHOR, M.M. Dispersion and disruption of tissue. In: Methods in Enzymology. Cell Culture. Academic Press, Inc., New York (In press)
3. BRINK, E.W., W.D.A. PERERA, S.P. BROSKE, R.A. CASH, J.L. SMITH, H.E. SAUBERLICH, AND M.M. BASHOR. Vitamin A status of children in Sri Lanka. (Submitted for publication)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
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A. PRIMARY	61101A	3A161101A91C	00	042			
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11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Long Term Cryopreservation of Platelets for Immediate Field Use							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 Clinical Medicine; 012900 Physiology; 008800 Life Support							
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME: Buchholz, William M., MAJ, MC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Zuck, Thomas F., LTC, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Platelet Storage; (U) Cryopreservation; (U) Blood Storage; (U) Massive Transfusion; (U) Platelet Transfusion; (U) Traumatic Hemorrhage							
23. TECHNICAL OBJECTIVE. <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Massive transfusion of stored blood following severe combat injuries leads to dilutional thrombocytopenia and increased bleeding secondary to the lack of platelets. Platelet transfusions can correct this defect, but conventional liquid platelet preservation is impractical for field use since platelets are not viable if stored for more than 72 hours. This study explores methods for freezing platelet concentrates so they may be preserved indefinitely and infused without time-consuming washing procedures.</p> <p>24. (U) Studies are in three areas: a) trials of combinations of cryoprotective agents and precise freezing and thawing rates which will satisfactorily preserve platelet function and viability; b) establishment of in vitro and in vivo animal tests which will predict clinical effectiveness in humans; and c) establishment of the conditions for preparing platelet concentrates which will maximize platelet yield and prevent the loss of large platelets, which are known to resist injury better than small platelets.</p> <p>25. (U) 76 10 - 77 09 a) In vitro testing of 124 different cryoprotective combinations have been performed, and 16 combinations seem satisfactory. After considerable delay, the liquid nitrogen freezer was acquired and tests performed to define and control the variables for reproducible freezing curves. Of the 16 cryoprotected platelet concentrates which were frozen at 2 C/min, 5 showed satisfactory post-thaw in vitro viability. b) In vitro platelet tests of metabolic integrity have been re-evaluated and a more sophisticated kinetic analysis of serotonin and chromium uptake is being undertaken. In vivo animal tests are being standardized prior to evaluation of frozen platelets. c) Platelet concentrates are routinely prepared to optimize platelet yield and preserve the fraction of large platelets.</p>							

<sup>a</sup> Available to contractors upon originator's approval

# ABSTRACT

PROJECT NO.	3A161101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	042	Long Term Cryopreservation of Platelets for Immediate Field Use

The following investigations are being conducted under this Work Unit:

- STUDY NO. 1 Cryopreservation strategies
- STUDY NO. 2 In vitro viability/function testing
- STUDY NO. 3 Nonhuman in vitro viability and function testing
- STUDY NO. 4 Maximization of platelet harvest

Massive transfusion of stored blood following severe combat injuries leads to dilutional thrombocytopenia and increased bleeding secondary to the lack of platelets. Platelet transfusions can correct this defect, but conventional liquid storage of platelets is impractical for field use since platelets are only viable when stored for less than 72 hours. Studies under this work unit explore methods for freezing platelet concentrates so that they may be preserved indefinitely and infused without time-consuming washing procedures.

Four in vitro tests have been reported in the literature to correlate with post-transfusion "viability" (the ability to circulate): serotonin uptake, morphology scores, chromium uptake, and osmotic shock resistance. After preliminary evaluation, the first 2 in vitro tests were chosen to evaluate platelet concentrates exposed to freezing and/or cryopreservation. One hundred twenty-four different combinations of penetrating and nonpenetrating cryoprotective agents have been tested. Sixteen of these showed satisfactory in vitro test results. These 16 have been frozen at a rate of 2 C per min to -80 C and then immediately thawed in a 37 C water bath. Five different cryoprotective agent combinations have shown satisfactory post-thaw in vitro platelet viability test results.

Preliminary evaluation showed serotonin uptake to be the most discriminating in vitro test. It became clear, however, that this test deserved a more in-depth study. The kinetics of serotonin uptake are being subjected to further study in an effort to characterize more fully serotonin uptake in fresh platelets and to gain further understanding into the types of lesions caused by cryopreservatives.

An in vivo animal model for testing platelet viability has been prepared and appears to be promising. Further testing has been limited by lack of personnel.

Platelet concentrates are now being prepared routinely so that the harvest of heavier and hemostatically more active platelets is maximized.



## BODY OF REPORT

WORK UNIT NO. 042

Long Term Cryopreservation of Platelets for Immediate Field Use

STUDY NO. 1

Cryopreservation strategies

### PROBLEM

Following massive transfusions for severe combat injuries, thrombocytopenia is the most common cause of generalized bleeding. While this bleeding may be prevented by platelet transfusions, conventional liquid storage of platelet concentrates is limited to 72 h at room temperature and to 24 h at 4 C. For field use outside of CONUS, these time limits are too restrictive to provide effective support for combat forces. The objective of this work unit is to develop strategies for preserving platelets indefinitely in the frozen state so that they may be infused immediately after thawing. Current methods of platelet cryopreservation employ cryoprotective agents that are toxic in the concentrations used and hence require extensive post-thaw manipulation and washing. This study seeks to exploit the synergistic effects of different nontoxic cryoprotective agents. All of the studies of this work unit relate to this objective.

### RESULTS AND DISCUSSION OF THE RESULTS

Combinations of up to 6 different cryoprotective agents were systematically evaluated. Their immediate toxic effect was assessed by measuring in vitro serotonin uptake rate and morphology scores. One hundred twenty-four combinations were tested, and 16 showed morphology scores greater than 70% and serotonin uptake rates of greater than 40% of fresh controls. These 16 were then frozen at a rate of 2 C per min to -80 C, and 5 showed satisfactory in vitro results. Other freezing rates remain to be tested. A major problem encountered with this phase was the irreproducibility of the freezing tracings. Considerable effort has been spent to standardize and control the multiple variables involved in freezing human platelets.

### CONCLUSIONS

Development of new combinations of cryoprotective agents require systematic empirical investigation. It is not possible thus far to predict the effect of adding or subtracting single agents to cryoprotective agent combinations.

### RECOMMENDATIONS

In vitro and in vivo "viability" tests should be improved (STUDY NO. 2). With these refinements, it should be possible to evaluate new combinations of cryoprotective agents and choose several with the greatest

promise. Platelets preserved with these cryoprotective combinations should be considered for testing in humans.

#### PUBLICATIONS

None

STUDY NO. 2

In vitro viability/function testing

#### PROBLEM

The objective of this study is to develop an in vitro system for assessing the metabolic integrity of cryopreserved platelets.

#### RESULTS AND DISCUSSION OF THE RESULTS

Four different methods for measuring in vitro platelet metabolic integrity have been evaluated and tested with fresh and damaged platelets: a) osmotic shock resistance, b)  $^{14}\text{C}$ -serotonin uptake, c)  $^{51}\text{chromium}$  uptake, and d) platelet morphology index. Serotonin uptake rate and morphology scores were chosen since they were the most reproducible. After testing the various cryoprotective agents, it became clear that an even more discriminating and sensitive test was required. Furthermore, these tests did not provide insight into the nature of the toxic effect on the platelets. Serotonin uptake was re-evaluated, and the conditions changed so that the kinetics of serotonin uptake could be fully characterized with a mathematical model. Serotonin uptake kinetics are now undergoing extensive investigation in order to understand and evaluate the in vitro toxicity from cryoprotective agents.

#### CONCLUSIONS

Chromium uptake and osmotic shock resistance are not useful because of erratic and irreproducible results. Measurement of serotonin uptake rate is highly reproducible and serotonin uptake kinetics are amenable to mathematical modeling. Serotonin uptake kinetics appear to be promising as an in vitro test of platelet metabolic integrity. Morphology score is a rapid procedure that can be used to screen combinations of cryoprotective agents for toxicity.

#### RECOMMENDATIONS

The kinetics of serotonin uptake should be evaluated further. Response of serotonin uptake kinetics to the metabolic effect of specific pharmacologic toxins should be evaluated. Ultimately, the utility of these in vitro tests to predict in vivo platelet survival should be evaluated in animal models and in man.



#### PUBLICATIONS

None

STUDY NO. 3

Nonhuman in vivo viability and function testing

#### PROBLEM

Conventional tests of platelet metabolic integrity and function correlate only roughly with clinical effectiveness. Animal models must be developed to verify the usefulness of cryopreserved platelets prior to any trials in man. The animal model proposed employs a rabbit first rendered thrombocytopenic and then exposed to a small wound in the jugular vein. Correction of the prolonged bleeding time and measurement of normal life span of transfused human platelets are the desired end points.

#### RESULTS AND DISCUSSION OF THE RESULTS

Preliminary work on this rabbit model has been completed. A procedure for performing jugular vein bleeding time has been developed. In non-thrombocytopenic animals, bleeding time is approximately 2 to 3 min and increases to greater than 30 min when the platelet count is less than 10,000/ $\mu$ l. Thrombocytopenia can be consistently produced by injecting platelet antiserum produced in a sheep. Busulfan has also been used to produce thrombocytopenia. The methods for producing reticuloendothelial blockade are not yet perfected and have caused death in several animals.

#### CONCLUSIONS

This rabbit model seems promising as an in vivo nonhuman model for platelet function. Thrombocytopenia can be produced but reticuloendothelial blockade requires further study.

#### RECOMMENDATIONS

This animal model should be evaluated further. A safe and effective system for blocking the reticuloendothelial system should be developed. Following this, fresh human platelets should be transfused and then their function and survival measured. After the function and survival of fresh human platelets are determined, cryopreserved platelet concentrates should be tested.

#### PUBLICATIONS

None

STUDY NO. 4

## Maximization of platelet harvest

### PROBLEM

Large platelets are heavier and more active metabolically and functionally than small platelets. The preparation of platelet concentrates involves differential centrifugation which may remove the large heavy platelets. To insure optimal platelet concentrates, specific preparatory methods must avoid the loss of large platelets and maintain platelet size distribution (PSD). In order to develop baseline data to determine accurately optimal preparation and storage manipulations, the effects of various anticoagulants on platelets from normal donors were investigated.

### RESULTS AND DISCUSSION OF THE RESULTS

No further work on this study has been done during this fiscal year. Table 1 in last year's report has been revised.

### CONCLUSIONS

Platelet concentrates prepared by centrifuging whole blood at approximately 660 x g for 8 minutes (first spin), and 3200 x g for 8 minutes (second spin) appear to be optimal. These procedures preserve the baseline PSD and insure both the optimum yield and function of the platelet concentrate.

TABLE 1  
Characteristics of the Platelet Size Distribution

	<u>Average*</u>	<u>Range*</u>	
Geometric Mean Cell Volume (fl)	5.82	4.78	7.46
Geometric Standard Deviation (fl)	1.73	1.64	1.83
Geometric Coefficient of Variation (%)	31.2	27.4	34.2
Arithmetic Mean Cell Volume (fl)	6.76	5.43	8.61
Arithmetic Standard Deviation (fl)	3.90	2.85	4.83
Arithmetic Coefficient of Variation (%)	58.0	52.2	62.0
Geometric Coefficient of Skewness	-0.012	-0.348	0.189
Arithmetic Coefficient of Skewness	1.540	1.048	1.920
Geometric Coefficient of Kurtosis	-0.113	-0.338	0.064
Arithmetic Coefficient of Kurtosis	3.109	1.009	5.715
Median Cell Volume (fl)	5.84	4.75	7.75
Megathrombocytes (%)	8.10	2.54	16.08
Thrombocrit (%)	0.160	0.096	0.222

\*Average and range for the parameters of the PSD in 43 healthy subjects. Blood was collected in balanced citrate.

### RECOMMENDATIONS

Baseline data developed in this study should be translated into the preparation and evaluation of techniques for preparing platelet concentrates for cryopreservation.

### PUBLICATIONS

1. BUCHHOLZ, W.M., and D.G. ODOM. Standardizing the investigation of platelet size distribution in humans. (Submitted for publication.)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6106	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61101A	3A161101A91C	00	043			
B. CONTRIBUTING							
C. <del>CONTRIBUTING</del>	None						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) The Effect of Gastrointestinal Hormones on Gastrointestinal Function							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry, 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 03		CONT		DA			
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING			
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		FUND (in thousands)	
C. TYPE:				77		.5	
D. KIND OF AWARD:				78		.5	
E. CUM. AMT.						18	
F. CUM. AMT.						27	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Hagler, L., COL, MC			
				NAME: Herman, R. H., COL, MC			
				POC: DA			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Combat Wounds, (U) Gastrointestinal Function, (U) Gastrointestinal Surgery, (U) Gastrin, (U) G.I. Enzymes, (U) Abdominal Injuries							
24. TECHNICAL OBJECTIVE <sup>a</sup> 25. APPROACH 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23.(U) Dietary substances cause changes in enzyme activities in the small intestine. Dietary substances cause the release of numerous hormones from the gastrointestinal (GI) tract. The GI hormone, gastrin, has a trophic action on the small intestine. In the absence of gastrin the small intestine atrophies and disaccharidases decrease in activity. It is possible that dietary substances affect small intestinal enzymes via the stimulation of gastrin. Healing of combat-incurred GI tract injury may be facilitated by the administration of gastrin.							
24.(U) The effect of gastrin on small intestinal enzymes of the partially gastrectomized dog will be studied. Appropriate analysis will be done to ensure that the animal is gastrin deficient. Various chemical forms of gastrin will be used to determine if small intestinal enzyme activity is increased. The response to dietary substances with and without gastrin will be tested. If gastrin is effective, other hormones of the GI tract will be tested.							
25.(U) 76 10 - 77 09 Studies demonstrate the feasibility of maintaining a partially gastrectomized dog in sufficiently good health so that tissue from the small intestine can be obtained on a periodic basis for analysis. If gastric resection is too drastic, the small size of the resulting stomach makes food intake insufficient to maintain weight. If too little of the stomach is removed, the animal will not become gastrin-deficient. A cutaneoduodenal fistula, created by mobilizing a segment of duodenum, has obviated problems which were encountered previously with a duodenal cannula. A method of repeated duodenal biopsy via the fistula has been applied successfully.							



# ABSTRACT

PROJECT NO.	3A161101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	043	The Effects of Gastrointestinal Hormones on Gastrointestinal Function

The following investigations have been conducted under this work unit:

STUDY NO. 1 In vivo study of the role of gastrin in the control of small intestinal mucosal enzymes on the dog

Gastrin, a polypeptide hormone, synthesized in the gastric antrum, has trophic effects on the small intestine in rats. Gastrin deficiency due to absence of enteral food or antral tissue leads to gastrointestinal atrophy. If the trophic effect of gastrin is required for normal intestinal protein synthesis, then gastrin deficiency (resulting from gastric or intestinal resection) will lead to abnormal protein and enzyme synthesis and subsequent abnormal gastrointestinal function. The acutely injured soldier with abdominal injuries requiring gastric or intestinal resection may be gastrin deficient and consequently postoperative gastrointestinal function may be favorably influenced by gastrin therapy. We plan to study the effects of gastrin on intestinal glycolytic enzymes in the dog made gastrin-deficient by antrectomy. Pilot studies are in progress to master the surgical techniques necessary to perform serial small intestinal biopsies. The major problem has been to learn how to antrectomize dogs so that they are sufficiently gastrin-deficient and yet can survive the operative procedure on a long-term basis.

## BODY OF REPORT

WORK UNIT NO. 043

The Effects of Gastrointestinal  
Hormones on Gastrointestinal Function

STUDY NO. 1

In vivo study of the role of gastrin  
in the control of small intestinal  
mucosal enzymes on the dog

### PROBLEM

An important aspect of acute gastrointestinal disease involves combat-related abdominal injury and its sequelae. Abdominal injuries occur frequently in any military operation and develop serious complications. In World War II, in one field hospital, wounds of the stomach comprised 416 of 3,154 cases (13%) of abdominal injuries. The fatality rate was 40%. Approximately 30% of the abdominal injuries consisted of wounds of the small intestine. Approximately 20% of the total number of injuries required partial resection of the gastrointestinal tract. Many patients with abdominal injuries will have altered gastrointestinal function secondary to resection of portions of the intestinal tract. With improved techniques of first aid, evacuation, blood replacement, surgery, and prophylaxis and treatment of infection, we can expect an increased number of combat-wounded soldiers to reach the postoperative period. At this point only general supportive measures are available and no specific therapy is known which can hasten healing and restore function of the gastrointestinal tract. Food intake, intestinal hormones and intestinal adaptation all make considerable contributions to the recovery process after intestinal resection. Several observations suggest that the antral hormone, gastrin, has trophic effects on the gastrointestinal tract. In rats, gastrin has increased  $^{14}\text{C}$ -leucine incorporation into protein,  $^{14}\text{C}$ -orotic acid incorporation into RNA, and  $^{14}\text{C}$ -thymidine incorporation into DNA. Gastrin trophic effects have been demonstrated in in vitro tissue cultures of rat gastric and duodenal mucosa. Pentagastrin (PG) stimulated epithelial cell growth, decreased cell doubling time, and decreased cell contact inhibition.

Two different laboratories have demonstrated the importance of food intake in regulating small intestinal enzymes. In rat, intravenous hyperalimentation decreased intestinal maltase and sucrase activities. Tissue gastrin fell concomitantly. The disaccharidases were restored to control levels by PG which suggests that gastrin may control intestinal disaccharidases. Both tissue gastrin and intestinal disaccharidases returned to normal after oral feeding. Previous studies in this laboratory have demonstrated increased activity of jejunal glycolytic enzymes in response to carbohydrate meals. Specific sugars caused adaptive changes in the enzyme most concerned with the metabolism of the specific substrate. In addition there was a generalized increase in enzyme activity attributed to calories alone. Since food intake influences gastrin and intestinal enzymes, and



since gastrin has documented trophic effects in the gut it is conceivable that gastrin has a generalized effect on protein synthesis in the gut. If the trophic effect of gastrin is required for normal intestinal protein synthesis, then gastrin deficiency states occurring as a consequence of gastric or intestinal resection could result in abnormal protein synthesis and subsequent maladaptation of intestinal enzymes. There is ample in vivo and in vitro support for a gastrin trophic effect. There is also evidence to suggest that food intake is important in determining the level of intestinal enzymes and the amount of tissue gastrin. The acutely injured soldier who has lost variable amounts of stomach and small intestine has reduced intestinal function by virtue of the surgical resection. The enzyme activities in the remaining gut are responsive to food intake and gastrin, both of which have been reduced by the surgical procedure. It is reasonable to believe that replacement of gastrin will restore intestinal enzymes to normal and hasten restoration of gastrointestinal function. We plan to study the role of gastrin in the control of selected intestinal enzyme activities in dogs. Dogs will be made gastrin deficient by antrectomy. We will determine if any alteration of the intestinal enzyme activity can be reversed by administration of exogenous hormones (gastrin G-17 and PG) or by endogenous small intestinal gastrin (ordinarily a minor source of the hormone) released by duodenal feeding of specific nutrients. These animal studies will help us design the appropriate studies of the effect of pentagastrin in patients (see Work Unit 011, Study No. 6).

#### RESULTS AND DISCUSSION OF RESULTS

Progress has been slow due to technical problems with the surgical preparation. Gastrectomy with jejunostomy and Roux-en-y anastomosis have been performed in three dogs. The first animal continued to leak around the cannula site, and so subsequent efforts were first directed at establishment of a mucous fistula. The second animal was sacrificed when it was impossible to control drainage of biliary secretions. At postmortem examination undigested food was noted in the colon so we believe that the animal starved from lack of digestive enzymes. Similar difficulties were encountered in the third animal, which died suddenly, one day after surgery which was performed to increase the length of the by-passed segment of gut. In June 1977, attempts were made to study the initial animal. We were in the second week of study when the biopsy device broke for the third time in 6 months.

#### CONCLUSIONS AND RECOMMENDATIONS

A new hydraulic pump should be purchased. Cannula maintenance has been satisfactory in the first animal but gentle manipulation is important. Dr. Rodkey, who is a veterinary surgeon in the Dept. of Surgery, is available to perform gastrectomies in future animals. The following schedule is recommended:

Period I      Days 0 - 7

1. Day 0      fasting after 0800 h.
2. Day 1      continue fast, biopsy duodenum.
3. Day 2      biopsy 0800, resume regular diet. \*
4. Days 2-7   begin intraduodenal powdered milk.

Period II      Days 7 - 9

1. Day 7      fasting after administration of powdered milk.
2. Days 8&9   duodenal biopsy at 0800 h on days 8 and 9 resume diet after biopsy on day 9.

Period III      Gastrin Infusion

1. Day 15      fasting after 0800 h; intravenous line placed at 1500 h.
2. Day 15      pentagastrin, intravenously 2 mg/kg/h for 16 h.
3. Days 16-18   duodenal biopsy.
4. Day 22      duodenal biopsy.

\*To give 20 g of protein, dilute 60 g of powdered milk in 75 ml water and give 62 ml to the animal.

PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMM'Y	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISB'N INSTR'N	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
N/A	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	61101A	3A161101A91C		00	044		
B. CONTRIBUTING							
C. CONTINUING	NONE						
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Influence of Stress and Environment on the Nutrition and Metabolism of Military Personnel							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002300 Biochemistry; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL		77	
C. TYPE				YEAR		1.5	
D. AMOUNT:				CURRENT		68	
E. KIND OF AWARD:				78		1.8	
F. CUM. AMT.						99	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Sauberlich, H.E., DAC			
				NAME:			
				POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Nutrition; (U) Military Medicine; (U) Metabolism; (U) Nutritional Alterations; (U) Environment; (U) Laboratory Animals; (U) Adaptation; (U) Endocrine							
23. (U) Evidence indicates that various stress factors and environmental exposures markedly alter the nutritional and metabolic patterns of mammalian species. Military personnel likewise are exposed to stressful states and environments in the performance of military duties. Fundamental studies will be conducted with the use of laboratory animals to identify the metabolic reactions and nutritional alterations that occur in response to a given stress or environment. The initial objectives include: (a) evaluate the biochemical and endocrine responses that occur with a stressful environment such as cold, calorie restriction, and adverse diets; (b) determine the degree of adaptation induced by dietary changes and environmental exposures; (c) investigate adverse effects of stress; (d) determine deleterious effects and interactions of various stresses such as dietary excesses, restricted nutrient intakes, cold, heat, etc., on physical and mental performance.							
24. (U) The studies will require the establishment of suitable laboratory animal models and the development of novel biochemical techniques. Various metabolic pathways and hormonal systems will be followed in response to nutritional alterations and stress conditions. Knowledge obtained from the fundamental studies conducted will provide guidance for applied investigations on the influence of stress and environmental factors on the nutrition and metabolism of military personnel.							
25. (U) 76 10 - 77 09 Glucagon enhances in vivo oxidation of C-6 glucose and has no effect on oxidation of C-1. Insulin stimulates oxidation of C-1 with no effect on C-6 of glucose. In addition, glucagon stimulates adrenal steroidogenesis. Epinephrine does not affect the rate of glucose oxidation.							

# ABSTRACT

PROJECT NO.	3A161101A91C	Military Internal Medicine
TASK NO.	00	Internal Medicine
WORK UNIT NO.	044	Influence of Stress and Environment on the Nutrition and Metabolism of Military Personnel

The following investigation has been conducted under this work unit during the past year:

STUDY NO. 1 In vivo enhancement of glucose oxidation  
by glucagon, epinephrine, or insulin

STUDY NO. 2 Glucagon and adrenal steroidogenesis

STUDY NO. 1. In vivo effects of glucagon, insulin, and epinephrine on glucose oxidation were studied in rats. The results indicate that glucagon stimulates oxidation of 6-<sup>14</sup>C-glucose and insulin enhances oxidation of 1-<sup>14</sup>C-glucose. In contrast, epinephrine did not significantly alter oxidation of glucose. Glucagon reversed the insulin effect on C-1-glucose oxidation. Insulin, however, failed to reverse the glucagon effect on C-6-glucose oxidation. The results indicate diverse effects of the two hormones on glucose utilization.

STUDY NO. 2. Intravenous administration of glucagon stimulates synthesis of corticosterone in the adrenal gland from fed rats. These findings are in accord with enhanced gluconeogenesis observed under a variety of nutritional and environmental stress situations.



## BODY OF REPORT

WORK UNIT NO. 044 Influence of Stress and Environment on the Nutrition and Metabolism of Military Personnel

STUDY NO. 1 In vivo enhancement of glucose oxidation by glucagon, epinephrine or insulin

### PROBLEM

The essential role of glucagon, insulin, and epinephrine in the moment-to-moment regulation of glucose homeostasis in mammalian species subjected to a variety of nutritional or environmental stresses has been recognized. Changes in plasma levels of these hormones have been observed during a period of food deprivation, during strenuous physical exercise, under various psychological stresses, or during an exposure to extreme environmental temperatures and terrestrial altitudes. In such situations, a proper distribution of metabolic fuels is essential for survival. Consequently, the role of the 3 hormones in mitigating harmful effects of stress as may be encountered in military combat is apparent.

In general, increased utilization of the catabolic pathways of glucose is followed by an increased production of carbon dioxide. Available evidence, however, indicates that glucagon may have divergent effects on rates of glucose oxidation by different tissues or organs. Glucagon reduces oxidation of U- $^{14}$ C-glucose both in perfused rat liver and in rat liver slices, but stimulates glucose oxidation in perfused rat heart. It has no effect on glucose oxidation in adipose tissue. In contrast to glucagon, insulin consistently stimulates glucose oxidation both in vitro and in vivo. Thus, insulin enhances oxidation of U- $^{14}$ C-glucose in rat epididymal adipose tissue; oxidation of C-1 of glucose is stimulated to a greater extent than oxidation of C-6. Administration of insulin to diabetic rats restores in vitro hepatic oxidation of 1- $^{14}$ C or 6- $^{14}$ C-glucose to the level observed in control rats. Insulin also stimulates oxidation of U- $^{14}$ C-glucose in rat diaphragm preparations. Exogenous insulin enhances in vivo oxidation of U- $^{14}$ C-glucose in fed or fasted rats and of 1- $^{14}$ C-glucose in fed rats. Epinephrine reduces oxidation of U- $^{14}$ C-glucose by liver slices from fed, fasted, or refed rats.

Beyond these observations little is known about the impact of the 3 hormones on glucose oxidation in the intact animal. It has been well established that glucagon or insulin administration evokes compensatory changes in systemic levels of several hormones and metabolites, which in turn, markedly alter a number of metabolic parameters. As a consequence, the rate of glucose oxidation observed in vivo represents the summation of such complex metabolic interactions. Since glucagon, epinephrine, and insulin control the major metabolic pathways of glucose utilization, pronounced effects of these hormones on in vivo oxidation of specific carbons of the glucose molecule would be anticipated.

## RESULTS AND DISCUSSION OF RESULTS

Male rats weighing 280-300 g were fed a complete casein-sucrose diet for 7 days. After this period of dietary adjustment, the rats were anesthetized with 50 mg/kg pentobarbital and 10 min later they were administered via the tail vein a solution of glucagon, epinephrine, or insulin.

Control rats were injected with the corresponding volume of the diluent. Five min thereafter a saline solution of  $^{14}\text{C}$ -glucose was injected into the tail vein (3.0  $\mu\text{Ci}/100$  g body weight). Immediately after the injection, the rats were placed in plastic bottles and expired  $^{14}\text{CO}_2$  was collected for 20 min in an aqueous solution of 2% NaOH. At the end of the collection period, radioactivity of the solution was determined.

Compared to the controls, glucagon doubled the oxidation rate of U- $^{14}\text{C}$ -glucose and insulin increased oxidation by about 68%. When administered in combination, glucose oxidation was increased by approximately 80%. In contrast, the effect of epinephrine on glucose oxidation was not statistically significant. Glucagon had no effect on oxidation of 1- $^{14}\text{C}$ -glucose. However, insulin enhanced oxidation by 70% over the control values. No insulin effect was observed when hormones were administered in combination, which indicates that glucagon reversed the insulin effect on C-1-glucose oxidation. Glucagon increased oxidation of C-6-glucose by 83% over the controls, but insulin had no effect. Glucose oxidation remained elevated after administration of both hormones. Thus, insulin failed to overcome the glucagon effect on C-6-glucose oxidation. Oxidation rate of 2- $^{14}\text{C}$ -glucose was almost doubled by glucagon or insulin.

## CONCLUSIONS

Increased formation of  $^{14}\text{CO}_2$  from C-6 of glucose relative to C-1 has been interpreted as increased activity of the citric acid cycle apparently as a consequence of incomplete equilibration of dihydroxyacetone-P with glyceraldehyde-3-P. Increased oxidation of C-1 of glucose induced by insulin suggests increased activity of the pentose cycle. These conclusions are consistent with the effects of the hormones on enzyme activity of the two cycles. The failure of insulin to reverse the glucagon effect on C-6-glucose oxidation indicates a diverse effect of these hormones on the Embden-Meyerhof pathway of glucose metabolism.

## RECOMMENDATIONS

Studies concerned with the effects of various hormones on glucose metabolic pathways in animals exposed to nutritional and environmental stress are recommended.

PROBLEM

The role of the adrenal gland in adaptive phenomena and the role of glucocorticoids in protective reactions to stress have been recognized. The conditions which optimize the production of the adrenal hormones should be clearly defined. The effect of glucagon on adrenal steroidogenesis remains essentially unknown. Several reports in the literature indicate that plasma levels of corticoids remain unchanged after glucagon administration. Other reports, however, indicate that glucagon increases or decreases the concentration of corticoids in the plasma. The variety of actions of glucagon observed in such studies apparently reflects a dependence on dose, method of administration of the hormone or antagonism by other hormones. Adenosine 3':5'-cyclic phosphate (cyclic AMP) as the intercellular mediator of glucagon action enhances steroidogenesis in adrenal tissue preparations. This evidence is not completely conclusive, since high concentrations of cyclic AMP must be used to elicit steroid production (situations not encountered in in vivo systems). Accordingly, in the following experiments steroidogenic responses to glucagon were induced in vivo and subsequent metabolic investigations were conducted in vitro.

RESULTS AND DISCUSSION

Male rats weighing about 300 g were fed a complete casein-sucrose diet for 7 days. After this period of dietary adjustment, the rats were administered via the tail vein a solution of glucagon (100 mg/100 g BW). Control rats were injected with the corresponding volume of the diluent. Twenty min later the rats were sacrificed, the adrenal glands were removed, quickly cooled in saline and quartered. The quartered adrenals were incubated for 2 h in a Krebs-Ringer medium containing glucose and  $^{14}\text{C}$ -progesterone. At the end of the incubation period, the tissue was homogenized and corticosteroids were extracted with methylene chloride and separated on the thin layer chromatography plates. Radioactive components were subsequently located by autoradiography and identified by co-chromatography with authentic reference compounds and by color or fluorescence reactions.

The results indicate that the adrenal tissue converted  $^{14}\text{C}$ -progesterone into 13 radioactive components. Compared to the controls, glucagon enhanced by approximately threefold the synthesis of corticosterone and twofold the synthesis of deoxycorticosterone, 17-hydroxyprogesterone and dehydroepiandrosterone. Similar results were obtained in preliminary experiments when glucagon was added to the incubation media. The remaining radioactive metabolites have not been identified as yet.

CONCLUSIONS

Intravenous administration of glucagon stimulates synthesis of at least 4 adrenal hormones, in particular, corticosterone.

### RECOMMENDATIONS

Additional studies should be conducted to delineate the effects of glucagon on enzyme systems involved in the synthesis of adrenal corticoids.

### PUBLICATIONS

1. KLAIN, G.J., and W.C. BELL. In vivo effect of glucagon on glucose oxidation. (Abstract) Fed Proc 36:1145, 1977
2. KLAIN, G.J. In vivo lysine oxidation and incorporation into tissue proteins by fasted-refed rats. Nutrition Reports International 14:507, 1976
3. KLAIN, G.J., F.J. SULLIVAN, K.S.K. CHINN, J.P. HANNON, and L.D. JONES. Metabolic responses to prolonged fasting and subsequent refeeding in the pig. J Nutr 107:426, 1977
4. KLAIN, G.J. In vivo effects of glucagon on fatty acid synthesis in fasted and refed rats. J Nutr 107:942, 1977
5. KLAIN, G.J., and J.P. HANNON. Differential response of rat brown and white adipose tissue to environmental or nutritional stress. Comp Biochem Physiol 58B:227, 1977
6. DOHM, G.L., A.L. HECKER, W.E. BROWN, G.J. KLAIN, F.R. PUENTE, E.W. ASKEW, and G.R. BEECHER. Adaptation of protein metabolism to endurance training. Increased amino acid oxidation in response to training. Biochem J 164:705, 1977
7. DOHM, G.L., G.R. BEECHER, A.L. HECKER, F.R. PUENTE, G.J. KLAIN, and E.W. ASKEW. Changes in protein synthesis in rats in response to endurance training. Life Sci 21:227, 1977



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6101	77 10 01	DD-DR&E(AR)636	
3. DATE PREV. SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'TN INSTR' <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM A. WORK UNIT
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		6 11 01 A		3A161101A91C		00 045	
B. CONTRIBUTING							
C. CONTRIBUTING		None					
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Development of a Model Host System of American Cutaneous Leishmaniasis							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 08		Cont		DA		C. In House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING			
B. NUMBER * Not applicable				FISCAL YEAR		77 3.8 77	
C. TYPE:				CURRENT		78 2 54	
D. KIND OF AWARD:				E. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME * Letterman Army Institute of Research				NAME * Letterman Army Institute of Research			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Moussa, M. A., LTC			
				NAME: Mellick, P. W., MAJ POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Protective immunity; (U) Infectivity, (U) Animal model (U) Leishmania braziliensis, (U) Leishmania mexicana, (U) Pathology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objective is to develop a model host system of American cutaneous leishmaniasis for better understanding of the pathogenesis, immunity, and transmission of the disease. Knowledge gained from these studies is essential to the support of investigations leading to the development of improved measures for the protection and treatment of military personnel serving in endemic areas overseas.							
24. (U) A model analogous to infection in man will be sought in animals infected with <u>L. braziliensis</u> and/or modified with chemical and biologic agents to effect resolution of lesions, onset of delayed hypersensitivity, and resistance to challenge infection subsequent to cure. Infections will be produced and monitored by standardized quantitative methods established in this laboratory.							
25. (U) 76 10-77 09 Treatment of <u>L. braziliensis</u> -infected hamsters with the immunostimulating agent, Levamisole, did not effect resolution of chronic parasitic lesions or modify the infection to parallel the self-healing lesions seen in man. However, low level <u>L. braziliensis</u> infection characterized by palpable, nodular, non-ulcerating lesions was achieved in the guinea pig. It is necessary to have large numbers of <u>L. braziliensis</u> amastigotes for ongoing studies. Attempts were made to produce large yields of <u>L. braziliensis</u> amastigotes by depressing the immune response of donor hamsters with cyclophosphamide. Although larger lesions were observed in treated hamsters, the variability of parasite burden rendered the results of this study inconclusive. Severe disease was observed in the first documented case of metastatic visceralizing <u>L. braziliensis</u> in a hamster. No shift in virulence was demonstrated in the isolated parasites, which suggests the importance of host immunity in containing the infection.							

<sup>a</sup> Available to contractors upon originator's approval

# ABSTRACT

PROJECT NO. 3A161101A91C

In-House Laboratory  
Research

WORK UNIT NO. 045

Development of a Model  
Host System of American  
Cutaneous Leishmaniasis

Treatment of Leishmania braziliensis-infected hamsters with the immunostimulating agent, Levamisole, did not alter the immune response and effect resolution of chronic parasite lesions to parallel the self-healing lesions seen in man. However, limited but self-healing L. braziliensis infections may have been accomplished in the guinea pig. Inoculation of  $10^7$  L. braziliensis amastigotes in the nose of the guinea pig produced low-level infections, characterized by nodular, non-ulcerating lesions. Lesions were palpable by 12 days and regressed after 21 days. Large yields of L. braziliensis amastigotes are necessary to ongoing studies. Attempts were made to produce large yields of L. braziliensis amastigotes by immunosuppression of the donor hamsters with the chemical immunosuppressive agent cyclophosphamide (CY). CY was given 24 hours prior to infection as a single intraperitoneal dose of 225-300 mg/kg. Although larger lesions were found in hamsters treated with doses of CY <300 mg/kg, variable parasite yields rendered the results of this study inconclusive. Severe disease, characterized by multiple metastatic cutaneous lesions with abundant parasites, was observed in the first documented case of visceralizing L. braziliensis infection in a hamster. No shift in virulence of the organism was demonstrated in cutaneous and visceral infections produced by the isolated visceral parasites. This observation suggests the importance of even modest host immunity in containing this infection.

## BODY OF REPORT

WORK UNIT NO. 045

Development of a Model  
Host System of American  
Cutaneous Leishmaniasis

### PROBLEM

The American cutaneous leishmaniasis are sand fly-borne parasitic diseases encountered by U. S. military personnel stationed in endemic areas of Central and South America. Due to the potential life-threatening nature of L. braziliensis, it is imperative to develop adequate diagnostic and preventive measures to insure the protection of personnel susceptible to infection. However, achieving these goals is dependent upon establishing an animal model system to support appropriate investigations.

Specific models of L. braziliensis infection are needed to evaluate the capacity of leishmanial antigens to elicit protective immunity, to elucidate mechanisms of protective immunity and, by using the natural vector, to study biologic transmission and its interruption. An ideal animal model for studies of protective immunity should be capable of effecting protection. This requirement would be fulfilled by the demonstration of resistance to homologous challenge infection following induced or spontaneous resolution of the primary lesion, thereby paralleling the uncomplicated disease in man. An economical animal model displaying these characteristics has not been described.

Obtaining large numbers of L. braziliensis amastigotes for ongoing studies is difficult owing to the low-level immune response of the hamster. The parasite itself, however, is capable of causing severe infection characterized by lesions with large numbers of organisms. This finding emphasizes the need to evaluate the ability of selected immunosuppressive agents to increase parasite yields in donor animals.

### RESULTS AND DISCUSSION OF RESULTS

Our previous studies achieved reproducible L. braziliensis and L. mexicana infections in hamsters. These infections were characterized by dose-related quantifiable incubation periods and growth of lesions. Thus, an essential step was completed with the development of this infection model in which the effects of experimental variables can be defined. The quantitative methodology obtained from these studies is being applied to the development of a model to investigate protective immunity in L. braziliensis infections.

In the hamster, L. braziliensis lesions do not spontaneously resolve and show no trend toward resolution, which limits the use of this animal. However, the presence of a partially effective



immune response which contains, but does not eliminate, the infection is suggested by the cessation of lesion growth and onset of delayed hypersensitivity. Modification of this immune response to induce healing was attempted with the immunostimulating agent, Levamisole, given at a recommended dose of 2.5 mg/kg in several dosage schedules following infection. No effect of this treatment regimen was evident, as judged by the comparable onset, growth, and lack of resolution of lesions in treated and untreated hamsters.

Guinea pigs infected with L. donovani or L. enriettii eliminate these infections and develop a high degree of protective immunity. These findings suggested using the guinea pig to establish L. braziliensis infections. In one experiment, 5 guinea pigs were inoculated with  $10^7$  L. braziliensis amastigotes in the nose. Slight swelling was observed by 5 days after infection and nodules were palpable by 12 days. Pronounced erythema was noted in only 1 of the 5 animals. Ulceration did not occur and lesions began to regress by day 21. These observations indicate that the guinea pig can be developed further as a model to investigate protective immunity in L. braziliensis infections.

A similar approach was taken with inbred CBA mice which are susceptible to infection with L. tropica and L. donovani, are able to resolve these infections, and are resistant to challenge. Investigations to determine if this mouse strain produces a parallel syndrome with L. braziliensis are currently in progress.

It is not economically feasible to obtain large numbers of L. braziliensis amastigotes from donor animal lesions for use in infection, antigen production, and other ongoing studies. Immunosuppression of the host immune response presents a viable approach to this problem. Cyclophosphamide (CY), an immunosuppressive drug, was investigated for its possible effect on increasing yields of amastigotes from donor hamsters. Since the drug is an alkylating agent and mutagenic, it was given 24 hours prior to infection to avoid direct effects on the inoculated parasites. Doses of 225 mg/kg to 300 mg/kg (the approximate  $LD_{10}$ ) were utilized in several experiments. Lesions in hamsters treated with doses of CY <300 mg/kg grew faster and were larger than those in untreated hamsters. However, a consistent increase in yields was not obtained, nor was there a correlation between lesion size and parasite burden. When increased yields were obtained, they were not dose related and were of a low order of magnitude (2X-6X). While such results suggest a low-level effect, the variability in parasite yields rendered the results of this study inconclusive.

One saline-treated control hamster from the Levamisole study provided the first documented instance of an experimental L. braziliensis infection visceralizing from a primary cutaneous lesion. Necropsy findings resembled those of L. donovani infection in the hamsters:



abundant parasites were found in the spleen and, to a lesser degree, in the liver and bone marrow. Amyloid was present in the kidney, spleen, adrenal, and liver. Cutaneous lesions metastatic from the primary site on the flank were found on the back, nose, and all four paws, and contained abundant parasites. However, growth of cutaneous lesions from inoculation of this isolate and its growth in the viscera of other hamsters was found to be entirely comparable to the parent strain. These findings indicate the metastatic infection did not result from an adaptation or mutation of the parasite and suggest an immunologic deficiency in the host. Since L. braziliensis infection is normally contained but not eliminated in the hamster, these results stress the importance of even a partially effective host immune response in control of this parasite.

### CONCLUSIONS

The principal criteria of an ideal animal model suitable to investigations of immunity to L. braziliensis infection were established. The model must manifest resistance to challenge infection subsequent to spontaneous or induced resolution of the primary lesion (thereby paralleling uncomplicated human infection). Spontaneous resolution of L. braziliensis lesions in the guinea pig provided evidence to warrant further investigations of this animal. Levamisole, an immunostimulating agent, was ineffective in enhancing the immune response of hamsters to effect resolution of chronic L. braziliensis lesions. Other agents with different modes of action, such as BCG, remain to be investigated. The importance of even a partially effective host immune response in controlling L. braziliensis infection was shown in a hamster in which deficient immunity resulted in severe metastatic disease. These findings emphasize the value of immunosuppression as an approach to achieving increased yields of L. braziliensis amastigotes from donor hamsters and to providing a model to study the more severe forms of the disease. Variable parasite yields rendered the results obtained with one immunosuppressant, cyclophosphamide, inconclusive.

### RECOMMENDATIONS

Emphasis should be given to the least complicated approaches in developing an animal model to study protective immunity to L. braziliensis infection. Based on the finding of spontaneous resolution of L. braziliensis lesions in the guinea pig, investigation of this animal as a potential model should be pursued. Efforts should be made to establish protective immunity by therapeutic modification of chronic L. braziliensis infection in hamsters. This approach should be pursued to provide an alternate model to study immunity in those more serious and chronic forms of cutaneous leishmaniasis. Since abundant parasites were found in lesions of an L. braziliensis-infected hamster with deficient immunity, studies with immunosuppressive agents should continue in order to obtain increased yields of

parasites to meet research requirements. In these studies, emphasis should be placed on agents such as antilymphocyte serum which can be given following infection to maintain an immunosuppressed state.

#### PUBLICATIONS

WILSON, H. R., and D. G. FAIRCHILD. Modified Whipf's polychrome: A connective tissue stain with special application for demonstrating Leishmania. Stain Technol 52: 105-111, 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)6.16	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DES'N INSTR <sup>a</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM A. WORK UNIT
77 07 19	H.Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61101A	3A16110A91C	00	051			
B. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Influence of Shock on Excretory Function of the Liver							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 012900 Physiology; 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 08		77 09		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES EFFECTIVE:				PREEXISTING		B. FUNDS (In Thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		77	
C. TYPE				CURRENT		1.0	
D. KIND OF AWARD				78		0.0	
E. CUM. AMT.						38	
F. CUM. AMT.						00	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Div of Combat and Experimental Surg			
				Department of Surgery			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. ABSTRACTS (Precede EACH with Security Classification Code) <sup>a</sup> (U) Hypovolemic Shock; (U) Bile Salt Secretion (U) Bile Salt Synthesis; (U) Liver Excretory Function; (U) Bile Secretory Rates							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) <sup>a</sup> 23. (U) Deterioration of the excretory function of the liver not uncommonly complicates the recovery of the wounded soldier, and frank liver failure may result. Bile salt secretion is one of the most sensitive parameters of the excretory function of the liver. The effects of hypovolemic shock on hepatic synthesis and secretion of bile salt will be investigated, and resuscitative agents and measures to modify adverse effects will be tested.							
24. (U) A simplified model, to allow long-term measurement of hepatic bile salt synthesis and secretion in baboons with intact gallbladders, will be used. These will be assessed during normovolemic control periods and during and after hypovolemic shock. A stream-splitter pump, returning 95% of collected bile in a chronic biliary fistula, will allow continued sampling of bile without interruption of the enterohepatic circulation (EHC).							
25. (U) 76 10 - 77 09 In fasting normovolemic baboons, the liver secretes 569±57 μmols of bile salt per hour; 43±6% of this enters the gallbladder and the rest circulates through the EHC in 75±16 minutes. The liver synthesizes 9.6±1.2 μmols of bile salt per hour and maintains the total bile salt pool at 2100±169 μmols, of which 1720±130 μmols is stored in the gallbladder during fasting. During hypovolemic shock (systolic BP less than 60 mm Hg), bile salt synthesis by the liver is reduced to 15% of control values, but bile salt secretion is unaffected. These changes are transient, and reverse (to normal) within 12 hours of resuscitation. Studies were terminated with the departure of the principal investigator from the Army.							

# ABSTRACT

PROJECT NO.	3A161101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	051	Influence of Shock on Excretory Function of the Liver

The following investigations have been conducted under this Work Unit:

A subhuman primate model (baboon) was developed under this work unit that allows a number of measurements of liver function to be made under completely physiologic conditions.

The enterohepatic circulation (EHC) is maintained and the gallbladder functions normally. The baboon model permits measurement of a) hepatic secretion rates of bile salt, cholesterol, and phospholipids; b) distribution of hepatic bile production between the gallbladder and the EHC; c) gallbladder function (percent gallbladder filling and evacuation); d) EHC circulation time; e) bile salt synthetic rate; and f) total and circulating (effective) bile salt pool size.

In chronically maintained male baboons, fasted, with a controlled bile fistula, the liver secretes  $569 \pm 57$   $\mu$ moles of bile salt per hour;  $43 \pm 6\%$  of this enters the gallbladder and the rest circulates through the EHC in  $75 \pm 16$  minutes. The liver synthesizes  $9.6 \pm 1.2$   $\mu$ moles of bile salt per hour and maintains the total bile salt pool at  $2100 \pm 169$   $\mu$ moles, of which  $1720 \pm 130$   $\mu$ moles are stored in the gallbladder during fasting.

In response to a meal, the gallbladder evacuates  $83 \pm 16\%$  of its contents over a 110-min period, and hepatic secretion of bile salt increases to  $1284 \pm 250$   $\mu$ moles per hour. These studies provided the necessary baseline values to which similar measurements during hypovolemic shock could be compared.

During hypovolemic shock (systolic BP less than 60 mm Hg), bile salt synthesis by the liver is reduced to 15% of control values, but bile salt secretion is unaffected. These changes are transient and reverse to normal within 12 hours of resuscitation.



## BODY OF REPORT

WORK UNIT NO. 051

Influence of Shock on Excretory  
Function of the Liver

### PROBLEM

Severe injury and hypovolemic shock resulting from combat trauma are associated with a significant incidence of multiple organ failure. Massive metabolic insults mediated through the effects of shock and trauma, as well as multiple transfusions, stress the excretory function of the liver. Deterioration of liver function can complicate the patient's clinical response; frank liver failure may result.

Our long-range goals are to investigate the effects of blood loss, trauma, and multiple transfusions on the excretory function of the liver and, ultimately, to determine means of improving the liver's response to shock and stress. Since bile salt secretion is one of the most sensitive indicators of the excretory function of the liver, it has been chosen as the major primary variable for investigation. Study of bile salt metabolism is complicated by the enterohepatic circulation (EHC), which, among other things, is affected by the presence of a functioning gallbladder. It was necessary to perfect a primate model which permits the necessary measurements of bile salt metabolism to be made without interrupting the EHC or interfering with normal gallbladder function. This has been accomplished in a baboon model under this Work Unit.

The goal of this study was twofold: (1) to conduct a series of baseline measurements using the model (the measurements can then be used as a standard of reference during experimental periods); and (2) to use the model to determine what effect shock and multiple transfusions have on liver function.

### RESULTS AND DISCUSSION OF THE RESULTS

Five male baboons were prepared, each with a duodenal fistula. Bile from the fistula was passed through an electronic stream-splitter. Five percent of the total bile flow was continuously sampled by the stream-splitter, and the remaining 95% returned to the animal through a tube duodenostomy. In this way, bile flow could be measured and samples collected without interfering with the normal EHC or normal gallbladder function. To assess bile salt secretion rates and bile salt pool size, bile salt labeled with  $^{14}\text{C}$  was given to the animals. Bile salt synthesis was measured directly by the "wash-out" technique of Small. Initial technical problems with the model have been overcome, essentially, and the procedure appears to be well tolerated by the animals.

To define bile salt metabolism, each animal was studied immediately following a fatty meal, and again after a 12-h fast. Four parameters were measured: a) percent of hepatic bile entering the gallbladder; b) rate of bile salt secretion from the liver; c) size of bile salt pool and the amount of this pool sequestered in the gallbladder; and d) percent of gallbladder contents evacuated in response to a fatty meal.

Results are expressed as the mean of 15 experiments ( $\pm$ SEM), 3 in each of 5 animals. Immediately following a fatty meal, there is a total bile salt pool of  $2100 \pm 169$   $\mu$ moles of bile salt, all of which is circulating within the EHC. The liver secretes  $1284 \pm 250$   $\mu$ moles of bile salt per hour. None of this bile salt enters the gallbladder, and it circulates through the EHC in  $75 \pm 16$  min. The bile salt pool is maintained by a de novo synthesis of  $9.6 \pm 1.2$   $\mu$ moles of new bile salt per hour. After a 12-h fast, the gallbladder begins to sequester a significant portion of the circulating bile salt pool, so that only  $380 \pm 52$   $\mu$ moles of bile salt remains circulating in the EHC. The rest is stored within the gallbladder. Since the amount of bile salt circulating in the EHC is reduced, bile salt secretion from the liver falls to  $569 \pm 57$   $\mu$ moles per hour. Of this bile salt,  $40 \pm 9\%$  ( $228 \pm 72$   $\mu$ moles per hour) enters the gallbladder, and the rest passes directly into the duodenum to enter the EHC. The gallbladder evacuated  $83 \pm 16\%$  of its contents over a 110-min period in response to a fatty meal, but no bile salt leaves the gallbladder during a fasting period.

In 4 animals, the effects of hypovolemic shock on these parameters were measured. Animals were acutely bled through a central venous catheter at 15 cc/min until systolic blood pressures between 50-60 mm Hg were obtained. During this period, bile salt synthesis is reduced to 15% of control values, and in one animal ceased completely. Bile salt secretion from the liver, as opposed to bile salt synthesis, is unaffected, even by profound hypovolemia, nor is the proportion of hepatic bile that enters the gallbladder affected. The effect of hypovolemic shock on bile salt synthesis is relatively transient. Within 12 h of returning the shed blood to the animals, bile salt synthesis is 80% of normal, and by 24 h it is back to control values.

#### CONCLUSIONS

This baboon model allows not only the comprehensive study of bile salt metabolism under completely physiologic conditions but could be used to investigate any aspect of hepatic secretory physiology, i.e., bilirubin metabolism, secretion of drugs, blood preservatives, etc. The procedure has been well worked out, and initial problems with the model and the animals' maintenance have been overcome. An extensive series of baseline values have been accumulated in intact fasted and fed animals, and these have been compared to a pilot group of studies in hypovolemic animals.

#### RECOMMENDATIONS

Study of the effects of hypovolemic shock on liver function has been discontinued because the principal investigator has left the Army. The model that has been perfected could be of great use to any investigator interested in the excretory function of the liver.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISB'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
76 10 01	H. Termination	U	U	DA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61101A	3A161101A91C	00	060			
B. CONTRIBUTING							
C. <del>CONTRIBUTING</del>	NONE						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Vitamin D, Calcium and Phosphorus Metabolism (06)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry; 002600 Biology; 0016800 Toxicology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
71 04		77 10		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		76	
C. TYPE:				CURRENT		1.8	
D. KIND OF AWARD:				78		0.0	
E. CUM. AMT.						00	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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TELEPHONE: 415 561-3600				SOCIAL SECURITY ACCOUNT NUMBER			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Applicable				NAME: Zolock, D.T., CPT, MS			
				NAME:			
				POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>							
(U) Combat Bone Injuries; (U) Vitamin D; (U) Calcium; (U) Phosphorus; (U) Mineral Metabolism							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Metabolic effects of vitamin D<sub>3</sub> and its metabolites include induction of calcium binding protein (CaBP) in intestine and kidney and increased intestinal calcium absorption. Bone and joint injuries account for approximately 25% of the operations performed at combat surgical hospitals. At current hospitalization costs (\$168.00/day) and a conservative estimate of \$20.00/day for soldier compensation, the bone and joint injuries treated in the Army between 1964 and 1969 would cost \$935,008,282.30. Also, in FY 75, Army dental facilities performed 859,722 tooth restorations at a cost of \$6,488,290.00. It is anticipated that nutritional and medical therapeutic improvements resulting from this research may improve these statistics, through improved absorption and retention of calcium and phosphorus.</p> <p>24. (U) The influence of nutritional factors (i.e. dietary calcium, phosphorus and vitamin D) on the intestinal CaBP concentration and calcium binding activity was studied in animal models. Also, in vitro and in vivo animal studies were performed to define better the mechanism of calcium transport and homeostatic regulation.</p> <p>25. (U) 76 10 - 77 09 Bone densitometry has been performed on several (27 determinations) patients referred to us from Letterman Army Medical Center. They had diagnosed or suspected metabolic bone disease. Also, several in vitro and in vivo animal studies have been designed and completed, resulting in regulation of calcium absorption and retention. Technological advances from this work unit have been reported in 11 research journal articles (published or in press), 10 published abstracts, 9 articles submitted to publishers and 7 potential manuscripts currently in an advanced stage of laboratory analysis of data.</p>							

<sup>a</sup> Available to contractors upon originator's approval.

DD FORM 1498  
1 MAR 66

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.



# ABSTRACT

PROJECT NO.	3A161101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	060	Vitamin D, Calcium and Phosphorus Metabolism
STUDY NO.	9	Kinetics and localization of calcium binding protein appearance in chick intestine following 1,25-dihydroxy-cholecalciferol administration

Results of a series of experiments designed to assess the acute response of rachitic chick intestine to 1,25-dihydroxy vitamin D<sub>3</sub> (1,25 OH<sub>2</sub>D<sub>3</sub>) have demonstrated the following facts. 1) Calcium binding protein (CaBP) is localized inside intestinal epithelial cells. 2) The rate of calcium transport is increased before CaBP appears. 3) Cycloheximide blocks the synthesis of CaBP but does not prevent the increase in calcium transport. 4) 1,25 OH<sub>2</sub>D<sub>3</sub> increases the cellular accumulation of calcium initially. 5) At the onset of CaBP synthesis, calcium accumulation begins to decrease. If CaBP synthesis is blocked, this decrease does not occur. 6) Study of tissues fixed in osmium-pyroantimonate by electron microscopy demonstrates that the number of mitochondrial granules corresponds to the cellular accumulation of calcium. Therefore, CaBP appears to be nonessential to the initial induction of calcium transport by 1,25 OH<sub>2</sub>D<sub>3</sub>, but it is essential to the cell's ability to regulate intracellular calcium. Interpretation of these experiments has changed substantially our working hypothesis for intestinal absorption of calcium. CaBP, which was formerly thought to reside outside of the cells and accumulate calcium at the brush border, is now believed to reside inside the cell and must maintain low intracellular calcium concentration. However, CaBP is not essential for the initial transport of calcium. Further, these experiments strongly suggest that the increase in cell permeability to calcium mediated by 1,25 OH<sub>2</sub>D<sub>3</sub> occurs without participation of the nucleus and some of the changes requiring nucleus participation may be the result of increased calcium permeability rather than the cause of it.

## BODY OF REPORT

WORK UNIT NO.	060	Vitamin D, Calcium and Phosphorus Metabolism
STUDY NO.	9	Kinetics and localization of calcium binding protein appearance in chick intestine following 1,25-dihydroxy-cholecalciferol administration

### PROBLEM

Approximately 25% of combat trauma includes fracture wounds. The impact of fracture wounds is greater than that of flesh wounds because the degree of incapacitation is considerably greater, the patients usually must be evacuated, and the recovery time is usually prolonged. Fracture wounds accounted for 5.78 million man-days lost by the Army between 1964 and 1969. While considerable effort has been expended to develop and perfect methods of treatment for both flesh and fracture wounds, no specific efforts have been made at the field level to modify the susceptibility of soldiers to fracture wounds. Susceptibility to fracture wounds varies greatly. Some individuals are quite resistant even to heavy trauma. Others sustain fractures without apparent trauma (stress fractures). The degree of bone mineralization is a function of both the amount of calcium and phosphorus absorbed from the gut and that retained by the kidney. Vitamin D is intimately involved in both intestinal absorption and renal retention of calcium as well as in the mineralization process. To understand better the regulation of intestinal calcium absorption, the acute response to 1,25-dihydroxy vitamin D<sub>3</sub> (1,25 OH<sub>2</sub>D<sub>3</sub>) was studied in the intestine of chicks with rickets. We also evaluated the effect of various treatment regimens such as dosage of 1,25 OH<sub>2</sub>D<sub>3</sub>, dietary calcium and dietary phosphorus on intestinal transport of calcium, intracellular accumulation of calcium, and cell morphology.

### RESULTS AND DISCUSSION OF RESULTS

Calcium-binding protein (CaBP) synthesis in rachitic chick intestine is increased by 1,25 OH<sub>2</sub>D<sub>3</sub>. Previous studies have suggested that vitamin D<sub>3</sub> enhances polysomal-associated CaBP-specific mRNA two- to threefold. This increase in CaBP synthesis could be due to increased cytoplasmic CaBP mRNA or to an increased association of polysomes and CaBP mRNA. To distinguish between these possibilities and to study the site of regulation, cytoplasmic RNA was isolated from rachitic and 1,25 OH<sub>2</sub>D<sub>3</sub>-repleted chick duodena by phenol chloroform extraction of cytosol and purified by sequential treatments with LiCl, pronase, LiCl and ethanol. Purified RNAs (A<sub>260</sub>/A<sub>280</sub> = 2.0) were translated in a cell-free system derived from wheat germ. Protein synthesis, as monitored by <sup>35</sup>S-methionine incorporation, was linearly related to the amount of RNA added (125 mg RNA/ml).

Translation products were evaluated in three ways. First, by SDS-polyacrylamide gel electrophoresis (SDS-PGE), only a few differences between the products synthesized by mRNA from control and 1,25 OH<sub>2</sub>D<sub>3</sub>-treated chicks were found. Thus, the 1,25 OH<sub>2</sub>D<sub>3</sub> effect is specific for only a few proteins. Secondly, the products were immunoprecipitated by antibody to chick duodenal CaBP and the immunoprecipitates were analyzed by SDS-PGE. Samples from 1,25 OH<sub>2</sub>D<sub>3</sub>-repleted chicks showed only one band of radioactivity (at least fivefold in excess of rachitic samples) co-migrating with authentic CaBP. Finally, when the products were evaluated by Ouchterlony methods, results were identical to immunoprecipitation SDS-PGE. Parallel experiments with albumin antibody revealed no radioactive bands on SDS-PGE. These experiments indicated that 1,25 OH<sub>2</sub>D<sub>3</sub> enhances cytoplasmic levels of mRNA specific for CaBP and appears to regulate a subset of specific genes.

Rachitic chicks were injected intramuscularly with 62.5 pmol of 1,25 OH<sub>2</sub>D<sub>3</sub>. Cycloheximide (cyclo) in 20 µg doses was given intraperitoneally starting one h before 1,25 OH<sub>2</sub>D<sub>3</sub> administration, at 4-h intervals for the first 12 h, and at subsequent 6-h intervals. Calcium transport as measured by serum <sup>45</sup>Ca was elevated at 4 h, peaked at 8 h and then decreased in the 1,25 OH<sub>2</sub>D<sub>3</sub> chicks. In 1,25 OH<sub>2</sub>D<sub>3</sub>-cyclo chicks, serum <sup>45</sup>Ca was elevated at 6 h and continued to rise throughout the 24-h experiment. Calcium transport as measured by removal of <sup>45</sup>Ca from the intestine continued to rise throughout the 24-h experiment in both the cyclo and non-cyclo treated 1,25 OH<sub>2</sub>D<sub>3</sub> chicks. Calcium accumulation in the mucosa of the 1,25 OH<sub>2</sub>D<sub>3</sub> chicks was increased at 4 h, peaked at 6 h, then declined to control (0 h) level by 24 h, while accumulation in the 1,25 OH<sub>2</sub>D<sub>3</sub>-cyclo chicks continued to rise. Rachitic chicks given cyclo without 1,25 OH<sub>2</sub>D<sub>3</sub> showed no appreciable difference from the controls. CaBP was detected in the 1,25 OH<sub>2</sub>D<sub>3</sub> animals beginning at 8 h. Cyclo inhibited 1,25 OH<sub>2</sub>D<sub>3</sub>-induced CaBP, but a trace amount of CaBP was detected in the 24-h group. It is apparent that calcium accumulation in the mucosa does not require CaBP and in fact is decreased as CaBP is synthesized. Since 1,25 OH<sub>2</sub>D<sub>3</sub> stimulated calcium transport in the absence of CaBP but at the expense of an increased calcium content in the mucosa, we believe CaBP acts to regulate intracellular calcium.

Earlier studies showed that low dietary calcium increases the concentration of CaBP in intestine of normal chicks by increasing the 1α,hydroxylase enzyme activity in the kidney so that more 1,25 OH<sub>2</sub>D<sub>3</sub> is made available. In the past, an equimolar relationship between 1,25 OH<sub>2</sub>D<sub>3</sub> and synthesized CaBP has been assumed. We used a 3 x 3 factorial design of dietary calcium and dietary phosphorus concentrations to evaluate the role of dietary calcium and dietary phosphorus in the response of gut to 1,25 OH<sub>2</sub>D<sub>3</sub> treatment (62.5 pmol) after 24 h. The raw data suggest that both dietary calcium and phosphorus markedly influence the gut response to the same dose of 1,25 OH<sub>2</sub>D<sub>3</sub> as evaluated by calcium transport, calcium accumulation, in vitro calcium uptake



and concentration of CaBP induced. Of particular note, is the observation that the amount of CaBP synthesized by rachitic chicks from a given dose of  $1,25 \text{ OH}_2\text{D}_3$  was directly related to dietary calcium instead of the inverse relationship observed in vitamin D repleted (normal) chicks. We had predicted this result based on our hypothesis that CaBP synthesis could be a result of increased cell permeability to calcium rather than a direct effect of  $1,25 \text{ OH}_2\text{D}_3$  in the nucleus. When laboratory analyses are completed, the data will be analyzed by a multiple regression analysis approach with the dependent variables being dietary calcium, dietary phosphorus, and dietary  $1,25 \text{ OH}_2\text{D}_3$ , and with the independent variables being in vitro calcium uptake, calcium transport as assessed by serum, calcium transport as assessed by removal from gut, calcium accumulation, CaBP concentration in intestine, bone  $^{45}\text{Ca}$ , serum calcium concentration, and serum phosphorus concentration.

#### CONCLUSIONS

Based on observations in our laboratory, we propose this model which illustrates some of the metabolic effects of  $1,25 \text{ OH}_2\text{D}_3$  and the possible role of CaBP in the intestinal cell. Actinomycin D studies suggest a direct effect on the synthesis of alkaline phosphatase. Other studies have shown that the mRNA for CaBP is absent in the rachitic state; thus, an effect of  $1,25 \text{ OH}_2\text{D}_3$  at the transcription level in the nucleus is also indicated. However, since the permeability of the cell to calcium is affected even when protein synthesis is inhibited, it is clear that a direct effect of  $1,25 \text{ OH}_2\text{D}_3$  on the membrane is also involved. We expect that most of the calcium accumulated in the cells would be accounted for by that associated with membranes and/or mitochondria. The localization of CaBP within the intestinal cells is consistent with its being loosely associated with intracellular membranes, including the outer membrane of mitochondria. The role of preventing the accumulation of calcium in the cell could be accomplished by either blocking the uptake of calcium by mitochondria or enhancing the removal from mitochondria. This effect of CaBP is suggested by both our studies and in vitro studies with isolated mitochondria reported by Hamilton and Holdsworth (Aust J Expt Biol Med Sci 53: 453-478, 1975). We suggest that CaBP is an intracellular protein in the intestinal mucosa, but that it is not required for calcium transport. We believe that one of its functions, or maybe its only function, is to protect intracellular organelles from calcium toxicity during calcium transport through the cell. Although the mechanism is still subject to further research, we do feel that it is an important observation that we can associate the presence or absence of CaBP to the capacity of the cell to maintain low intracellular calcium concentration only when CaBP is present.



## RECOMMENDATIONS

1. Bone densitometry and ultrasound evaluation of military inductees should be performed. Inductees whose bone density is in the lower or subnormal range should receive dietary supplementation with calcium and magnesium as well as specialized exercise training programs during their basic combat training. Candidates should be prevented from entering military service if their bone density value is subnormal on the basis that a high probability exists that they will be included in the population of individuals medically retired due to bone and joint problems.
2. A staff study on the feasibility and legal requirements to formulate a calcium and magnesium supplemented flour for distribution to military feeding facilities should be initiated.
3. Contingency plans should be made to issue supplementary magnesium (250 mg magnesium oxide orally per day) to troops moving from a temperate to a high intensity sunlight region. The purpose would be to minimize the effect of hypercalcuria and decrease the chances of urolithiasis. Also, opportunities should be sought to evaluate the efficacy of the procedure in field trials in conjunction with training operations.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
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22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup> (U) Digital Computers; (U) Data Base Management; (U) Data Files; (U) Biomedical Research Information; (U) Statistics							
23. (U) The objective is to design, implement, and document computer programs and program systems for the management of LAIR research data. These programs will (a) process the results of clinical and laboratory studies to derive their conclusions and to test applicability to specific military situations, (b) maintain and utilize effectively a repository of past research data for direct application to military problems and correlation with future approved military research, (c) evaluate data reported in the open literature to determine its applicability and to apply it to the military environment by correlation and transformation techniques, (d) support the requirements of privacy and the freedom of information acts, (e) provide data base and rapid analysis for information within the mission areas of LAIR in the event of mobilization.							
24. (U) General purpose computer programs will be used to the greatest extent possible. Where the unique information processing requirements of a specific research protocol cannot be met by available general purpose computer programs, special purpose programs will be developed.							
25. (U) 76 10 - 77 09. Special purpose programs were produced for military biomedical research projects including muscle stress analysis, mosquito repellent effectiveness, beta counter data reduction, and animal breeding. A system to control automated treadmill experimentation, developed under contract, has been implemented on a laboratory minicomputer. Interactive programs have been written to process the entry of food wholesomeness data and owl monkey demographic, hematologic and serum chemistry data. A program to convert data files established on the institute central minicomputer and unloaded for further processing off-site has been developed. Data bases have been established for owl monkey demographic, hematologic and serum chemistry and human cardiac patients.							



# ABSTRACT

PROJECT NO. 3M161102BS02

Basic Mechanisms of Recovery  
From Injury

WORK UNIT NO. 055

Design of Military Biomedical  
Research Information Systems

The following studies have been conducted under this work unit:

STUDY NO. 1 Data processing support of biomedical research  
(General support)

STUDY NO. 2 Direct computer support to LAIR departments

Data processing support is essential to biomedical research for the construction of mathematical models, the calculation of data transformation, graphical displays and data management. Some of these functions are necessary to meet the various constraints of Army regulations, freedom of information acts, good laboratory practices acts and others. Whenever possible these needs are met by acquisition and/or modification of standard programs or program packages. When not possible, unique programs are developed. The military relevance of raw data and hypotheses are tested by the application of mathematical and statistical algorithm. Their validity in the military environment is determined and correlation with variables unique to the military environment are examined.

## BODY OF REPORT

WORK UNIT NO. 055

Design of Military Biomedical  
Research Information Systems

STUDY NO. 1

Data processing support to  
biomedical research (General  
support)

### PROBLEM

The objective of this study is to provide software resources of a general nature to support a variety of applications that are processed on LAIR minicomputers and/or the remote data processing facility located at the Lawrence Berkeley Laboratory (LBL). Software resources to be provided include systems and programs that provide data acquisition, graphical display of data, and management and analysis capabilities considered useful to the military biomedical research conducted at LAIR.

### RESULTS AND DISCUSSIONS OF RESULTS

For general data processing support to LAIR investigators, several programs have been implemented that are considered useful for processing functions common to applied experiments.

#### Department of Information Sciences

Applications Division. At present only two institute data bases are managed by the Remote File Management System (RFMS) because the number of data base elements definable under that system is limited and interfacing data from RFMS to statistical analysis systems requires programming of report generators and programs to output and recode data. It has been determined by the LBL staff that the desirable generalized data base management and basic statistical analysis system, Scientific Information Retrieval (SIR), is not transportable to the LBL CDC 7600/6600 system. Support of data base management systems at LBL is also changing and remote users have been informed of the planned implementation of SYSTEM 2000 and discontinued support of RFMS. The question of generalized data base management on the LAIR ECLIPSE C/300 central minicomputer is appropriate for an increasing number of institute applications that are being processed on that machine. It is recognized that INFOS, at present not implemented under the Data General Advanced Operating System (AOS), remains a file management system and real data base management capabilities cannot be realized in that system.

#### Department of Nutrition

Bioenergetics Division. The Treadmill Automated System (TAS) has been implemented on the MODCOMP II minicomputer by LBL personnel and sample test experiments have been duplicated by members of the Bioenergetics Division and the Department of Information Sciences. The system

is designed to minimize reprogramming as treadmill experiments and hardware and information requirements change. The system is also designed to allow for simultaneous interactive support for nutrition surveys and it may be transported and operated at off-site locations.

Food Hygiene Division. A program has been developed for the CDC 7600/6600 computers at LBL to decode a tape containing data files copied from the Data General C/300 central minicomputer system. This program is used to transfer coded microbiological data files from the LAIR computer to the LBL computer for subsequent statistical processing and data base storage. Deblocking and recoding of the file to effect CDC 7600/6600 characterization is handled by the program.

Radioisotope Division. A program has been developed to handle the input of records from paper tape to form an easily reusable disk-based file image of the tape. The name of the permanent file to which the file is written is specified by the user. Extraneous characters and records are removed under program control in the process of transferring character string segments from tape to disk.

#### CONCLUSION

Functions common to data and information processing given in support of research activities are developed and maintained at less cost through utilization of general purpose systems and programs.

#### RECOMMENDATIONS

A study of SYSTEM 2000 when implemented at LBL should be performed and its usefulness evaluated. The availability of a generalized data base management system for implementation on the LAIR central minicomputer should also be determined.

STUDY NO. 2

Direct computer support to  
LAIR departments

#### PROBLEM

Advances in data processing techniques today offer to military biomedical investigators methods of information processing that satisfy a variety of data acquisition, management, and analysis functions. The objective of this study is to provide the research departments of LAIR with direct analysis, programming, and operational support at the project level to meet research requirements.

#### RESULTS AND DISCUSSION OF RESULTS

Dissimilar operating system features between the LBL and LAIR data processing environments now offer to LAIR investigators a variety of data processing capabilities never before available. Data acquisition

and analysis functions can be performed more responsively locally, while the management and analysis of larger data bases in a batch processing environment can be supported best at LBL.

Systems and programs designed for investigators who prefer "hands on" access to computer based information and data processing resources are being developed on LAIR minicomputers. A shortcoming of those systems, however, is that some applications require data management and statistical analysis support which is not available in-house at present. This type of support is available at LBL, but cannot be implemented on the in-house ECLIPSE C/300 system. Thus the transfer of data between the LAIR and LBL environments is becoming a more urgent and outstanding requirement.

Computer support in FY 77 to departments at LAIR has been directed more to development of systems and programs that are uniquely applicable to an experiment than to general purpose programs and systems. Programming to support data entry and conversational processing functions requires program control and data flow to parallel the order and structure of the data in a specific experiment. Program development at LBL has been directed to unloading several data bases previously managed under the remote Remote File Management System (RFMS) to establish files in a format acceptable for input to statistical packages.

#### Department of Biomedical Stress

Military Stress Division. Analysis of muscle stress data has been completed. An editor was programmed to scan the file. After editing and reporting, averages of data samples were calculated and established on file. More extensive analysis of the resultant file has been completed using the Statistical Package for the Social Sciences (SPSS).

#### Department of Comparative Medicine

Animal Resources Division. Data processing for the management and statistical analysis of demographic, hematologic, and serum chemistry data collected from animals in the LAIR owl monkey breeding colony has been developed. A data entry program (with extensive editing capability) and a report program have been designed and programmed and are now being tested and documented. The system was developed on the ECLIPSE C/300, and is used by investigators from a remote interactive keyboard/display terminal. All animal identification and hemogram records previously recorded in laboratory notebooks have been established on file and the entry serum chemistry records has been initiated.



Pathology and Comparative Study Division. To determine the availability of an in-house veterinary pathology data management system, an extensive literature search and inquiry of universities and research institutes maintaining such systems have been performed. At present, the Natural Language Retrieval System (NLRS), developed at the University of California at Los Angeles, seems to be the best system to edit, manage, retrieve, and analyze pathology data with a natural language processing capability. Requirements exist for investigators to process data entry and retrieval functions in a conversational mode at an interactive terminal.

#### Department of Dermatology

Cutaneous Infection Division. Files managed by the Dermatology Data Collection System (DDCS) containing data acquired in three year-long periods of collection of outpatient data have been reported and archived. Requirements to analyze statistically the acquired data have been outlined. Acquired data elements common to each of the years of data collection have been recoded to establish a single file of records for input to SPSS. Over 146,000 initial patient visit records are to be analyzed. Preliminary analysis has indicated the need for categorizing diagnostic codes within that file and a request for such a classification has been forwarded to investigators. Additionally, a manager program which reports and updates records in the DDCS system has been developed and documented.

Cutaneous Protection Division. A mosquito repellent effectiveness data base managed under RFMS has been archived, and requirements for multivariate statistical analysis of the data base with the use of SPSS have been developed. Logical entries of data in the RFMS data base are non-rectangular, thus for input to SPSS, linearization of the data base is required. A design document for programs required to reformat and recode approximately 4,100 repellent effectiveness measurements to create an SPSS compatible file is being assembled. Additionally, variables describing properties of insects and volunteers, information previously not collected in the RFMS data base, will be defined in the SPSS data base and are to be used in future data collection and analysis.

#### Department of Medicine

Metabolic Laboratory Division. Programs to store and analyze data acquired from the division's beta counter have been implemented and the system is in production. Input of paper tape for disk-based storage and standard curve and unknown samples analysis is now performed by the user from a remote terminal located in the division. A system required to manage and analyze patient medication intake and temperature response data is to be designed and programmed. Likewise, in-house hardware required to support the application is to be identified.

## Department of Nutrition

**Bioenergetics Division.** Energy expenditure data reduction and analysis programs have been converted from the LBL environment to the in-house MODCOMP II minicomputer. Signal error detection and the real-time display of monitored data have been programmed. Programmer and user documentation manuals for the Treadmill Automated System (TAS) must be developed as little formal documentation was developed when the system was created by LBL.

**Food Hygiene Division.** The function of microbiological data entry performed under program control at an on-line terminal has replaced batch processing of data input from cards. Recoding of data from laboratory reports to keypunch forms and keypunching of card records has been eliminated in the system. File transfer requirements from the ECLIPSE C/300 to LBL (only the data entry function is programmed at LAIR) are satisfied with the program described in Study No. 1. Development of a data editor resident in the LBL system has been completed. All required reportings of data contained in the 1976 data base have been completed including processing of 1,500 reportings received from the Centralized Food Preparation Facility (CFPF) at Ft. Lee, Virginia, a new contributor of information to the data base. Microfilm recorded histogram displays depicting the frequency of occurrence of varying bacteria content levels amount similar food class and item samples remain to be processed.

**Radioisotope Division.** Programs existing on the RCA 301 computer at USAMRNL developed to support beta counter data reduction and analysis have been converted to the ECLIPSE C/300 system with substantial modifications to allow program execution in a time-sharing environment. Through conversational processing, the entry of user supplied data has been simplified and data interfaces between programs in the system are now handled under program control transparent to the user. Requirements for data processing support to reduce and analyze gamma counter data have been specified, a system has been designed, and programming has been initiated on the ECLIPSE C/300 computer. Functionally, the system is designed to calculate standard curve coefficients with the use of any of four models: a logit-log model, a non-linear four-parameter model, a hyperbolic model, and an adjusted end points logit-log model. Optionally, the "best-fit" standard curve may be program selected.

## PUBLICATIONS

FOWLER, J.L., D.L. STUTZMAN, J.F. FOSTER, and W.H. LANGLEY. Selected food microbiological data collected through a computerized program. J Food Prot 40:3, 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6112	77 10 01	DD-DR&E(AK)656	
3. DATE PREV. SUMM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'D INSTR' <sup>a</sup>	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
A. PRIMARY	61102A	3M161102BS02	00		056		
B. CONTRIBUTING							
XX C. CANCELLING	CARDS 114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Biochemical Basis of Nutrition, Disease and Recovery in Military Personnel							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry; 003500 Clinical Medicine; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER* Not Applicable				FISCAL YEAR		77	
C. TYPE:				CURRENT		3.0	
D. KIND OF AWARD:				78		3.4	
E. CUM. AMT.						124	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME* Letterman Army Institute of Research				NAME* Letterman Army Institute of Research			
Presidio of San Francisco, CA 94129				Biochemistry Division			
ADDRESS*				Department of Nutrition			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME* Milne, D.B., DAC			
TELEPHONE: 415-561-3600				TELEPHONE: 415-561-5872			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Turnbull, J.D., CPT, MS			
				NAME: Askew, E.W., CPT, MS			
				POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Nutrition; (U) Metabolism; (U) Military Nutrition; (U) Military Medicine; (U) Laboratory Animals; (U) Nutritional Disorders							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Various nutrients have been implicated in wound healing, bone repair and integrity, prevention of various cardiovascular and degenerative diseases, adaptation to environment and stresses, muscle functions and energy metabolism. Objective is to conduct fundamental research directed toward present or potential military problems concerning nutrition and metabolism, and the role of diet in health, disease, infection, immune response, and recovery from injuries.							
24. (U) Through use of laboratory animals and tissue culture systems, the biochemical basis of the role of nutrients and related compounds will be investigated as they may relate to the prevention and treatment of injury and disease, and to the functioning of military personnel under various conditions. Included for study are interactions of nutrition in toxicology and parenteral nutrition, aspects of medicinal nutrition, and pharmacological effects of vitamins and other nutrients. Techniques will be developed and investigations conducted that will provide knowledge as to the requirements, metabolism, interactions, utilization, and functions of nutrients that may be utilized in applied studies in military medicine and ration development.							
25. (U) 76 10 - 77 09 In contrast to the skeletal muscle, the brain does not appear to adapt to exercise by increasing its enzymatic capacity for ketone body oxidation. This emphasizes the importance of maintaining plasma glucose levels during exercise. Salicylates had no effect on carnitine acetyltransferase. Salicylates stimulate fatty acid oxidation in skeletal muscle mitochondria by a mechanism unrelated to mitochondrial uncoupling. Silicon levels in skin and tendons are influenced by dietary silicon. Dietary silicon has little effect on <sup>45</sup> Ca uptake by the bone, and no effect on calcium absorption from the intestine.							

\*Available to contractors upon originator's approval.



#### ABSTRACT

PROJECT NO.	3M61102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	056	Biochemical Basis of Nutrition, Disease, and Recovery in Military Personnel

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Dietary control of energy metabolism
- STUDY NO. 2 Trace elements in wound healing, collagen metabolism, and bone metabolism

STUDY NO. 1. Three new experiments have been written and approved for study under this work unit. Experiment 1 is designed to test the efficacy of dietary linoleate and undecanoate as gluconeogenic energy sources for exercising rats. Experiment 2 is designed to evaluate the influence of exercise on enzymes of brain ketone body metabolism. In the third experiment, the effect of anatomical site and exercise on the turnover rate of various rat adipose tissue depots is being investigated. Research is currently underway in all three of these areas.

STUDY NO. 2. Studies have been initiated to investigate the roles of various trace elements in bone formation and connective tissue metabolism. Silicon apparently plays a role in connective tissue metabolism and may function in the bone formation process.



## BODY OF REPORT

WORK UNIT NO.        056                    Biochemical Basis of Nutrition,  
   Disease, and Recovery in Military  
   Personnel

STUDY NO.            1                    Dietary control of energy metabolism

### PROBLEM

The objective of this study is to obtain basic information on the potential of dietary modifications to enhance physical performance. Inherent in studies of this nature is the need for basic biochemical information on the energy pathways being investigated. We are attempting to answer the following questions: Will dietary modifications designed to elevate muscle and liver glycogen prolong endurance? Does the brain adapt to chronic exercise by decreasing its reliance on glucose as an energy source and increase its reliance on ketone bodies? Which storage source of fat energy is the most important during exercise, e.g., the fat stored in close proximity to the viscera, or that stored in greater bulk around the kidneys, or that stored in various subcutaneous sites? Answers to these questions will provide information needed to design diets to enhance physical performance required of personnel in military operations.

### RESULTS AND DISCUSSION OF RESULTS

Preliminary results of a portion of this study indicate that unlike skeletal muscle, the brain does not adapt to exercise by increasing its enzymatic capacity for ketone body oxidation. This emphasizes the importance of plasma glucose concentrations during exercise.

A final experiment was conducted on the effect of salicylates on fatty acid oxidation (from Work Unit 058). Salicylate had no effect on carnitine acetyltransferase; thus a potential site of action of this compound in its enhancement of fatty acid oxidation was eliminated. The results from this experiment indicate that salicylate stimulates fatty acid oxidation in skeletal muscle mitochondria by a mechanism unrelated to mitochondrial uncoupling.

### CONCLUSIONS

The stimulation of fatty acid oxidation by pharmacological levels of salicylate has been documented with skeletal muscle mitochondria. Several proposed mechanisms of action of salicylate in exerting this effect have been discredited; however, the exact mechanism remains to be elucidated.

### RECOMMENDATIONS

Research on the biochemical mechanism of action of salicylate in stimulating fatty acid oxidation will be continued at Fitzsimons Army Medical Center, Department of Internal Medicine by the former principal investigator, CPT R.E. Jones.

### PUBLICATIONS

1. ASKEW, E.W., A.L. HECKER, and W.R. WISE, JR. Dietary carnitine and adipose tissue turnover rate in exercise trained rats. J Nutr 107: 132, 1977
2. DOHM, G.L., A.L. HECKER, W.E. BROWN, G.J. KLAIN, F.R. PUENTE, E.W. ASKEW, and G.R. BEECHER. Adaptation of protein metabolism to endurance training. Increased amino acid oxidation in response to training. Biochem J 164:705, 1977
3. DOHM, G.L., G.R. BEECHER, A.L. HECKER, F.R. PUENTE, G.J. KLAIN, E.W. ASKEW, and C.P. SMITH. Changes in protein synthesis in rats in response to endurance training. Life Sci 21:189, 1977
4. ASKEW, E.W., A.L. HECKER, V.G. COPPES, and F.B. STIFEL. Cyclic AMP metabolism in adipose tissue of exercise trained rats. (Abstract) Fed Proc 36:1157, 1977
5. ASKEW, E.W., A.L. HECKER, V.G. COPPES, and F.B. STIFEL. Cyclic AMP metabolism in adipose tissue of exercise trained rats. Submitted for publication
6. ASKEW, E.W., G.L. DOHM, P.C. WEISER, R.L. HUSTON, and W.H. DOUB, JR. Supplementary dietary carnitine and lipid metabolism in exercising rats. Submitted for publication
7. JONES, R.E., E.W. ASKEW, A.W. MEIKLE, and A.L. HECKER. Effects of salicylic acid on fatty acid oxidation by rat skeletal muscle mitochondria. Submitted to LAIR Manuscript Review Committee
8. ASKEW, E.W., S.T. SCHUSCHERBA, J.P. BROWN, and A.L. HECKER. Observations on preadipocyte distribution patterns in rat adipose tissue. Submitted for publication

STUDY NO. 2

Trace elements in wound healing,  
collagen metabolism and bone  
metabolism.

#### PROBLEM

Many trace elements are involved in bone calcification and collagen or elastin metabolism and thus may be important in recovery from injury. Silicon, a mineral whose essential role has recently been described, has been implicated in bone formation and in connective tissue metabolism. The initial experiments under this study were designed to establish procedures for studying silicon metabolism and to obtain basic information regarding silicon distribution and function.

#### RESULTS AND DISCUSSION OF RESULTS

Marginal silicon deficiencies have been produced in rats maintained in a "trace element sterile" isolator for periods of 3 to 5 weeks, by feeding them a purified amino acid diet which contained less than 5 ppm silicon. Growth rates were significantly improved by the addition of silicon to the diet. The silicon contents of the skin and tendons were directly related to the level of dietary silicate. Silicon levels of other tissues, like muscle, heart, liver and kidneys were not affected by dietary silicate. This indicated that silicon may be involved in connective tissue, and possibly collagen metabolism.

Organically bound silicon, in the form of diphenylsilanediol, which significantly increased growth rates, influenced silicon contents of the liver and skin. This indicated that the organic silicon compound could have been metabolized by the liver before being used by the connective tissues.

Preliminary experiments were conducted to study the effects of dietary silicon on  $^{45}\text{Ca}$  metabolism, since silicon was found to be uniquely located at the site of active calcification in the bone; it was hypothesized that silicon is involved in the bone calcification process. Dietary silicate or mild silicon deficiency has no apparent effect on  $^{45}\text{Ca}$  absorption from the duodenum. Only a slight (but not significant) depression of  $^{45}\text{Ca}$  uptake by the femur 30 minutes after injection was observed in weanling rats whose mothers were maintained on low silicon diets some two weeks before parturition. A time course study is needed to confirm the above observations.

#### CONCLUSIONS

Silicon is apparently involved in connective tissue metabolism and may be involved in bone metabolism.

### RECOMMENDATIONS

1. Studies on the mechanism by which silicon acts on connective tissue metabolism and bone formation should be continued.
2. Studies on the effects of other trace elements on wound healing, connective tissue metabolism, and bone metabolism are necessary.
3. Studies on the roles of essential tissue elements on performance and muscle dysfunction should be initiated.

### PUBLICATIONS

1. MILNE, D.B., D.D. SCHNAKENBERG, and D.D. WALLACE. Evaluation of semipurified and purified diets for the laboratory rat. (Abstract) Fed Proc 36:1155, 1977
2. MILNE, D.B., D.D. SCHNAKENBERG, and D.D. WALLACE. An experimental diet which compares with stock diets in rat growth studies. Submitted for publication



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. ORIGIN INST <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	
76 10 01	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO A. WORK UNIT	
10. NO./CODES <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		61102A		3M161102BS02		00	
B. CONTRIBUTING						057	
C. ORIGINATOR		CARDS 114f					
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Nutritional Physiology in Health and Prevention of Injury in the Military							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
00350 Clinical Medicine; 012900 Physiology; 006500 Food; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				B. PRECEDING		B. FUNDS (in thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		4.0	
C. TYPE:				CURRENT		156	
D. KIND OF AWARD:				78		4.0	
E. CUM. AMT.						214	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				NAME <sup>a</sup> Letterman Army Institute of Research ADDRESS <sup>a</sup> Bioenergetics Division Department of Nutrition Presidio of San Francisco, CA 94129 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
RESPONSIBLE INDIVIDUAL NAME: Canham, J. E., COL, MC TELEPHONE: 415-561-3600				NAME <sup>a</sup> Johnson, H. L., DAC TELEPHONE: 415-561-5092 SOCIAL SECURITY ACCOUNT NUMBER			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Applicable				NAME: Schnakenberg, D.D., MAJ, MS NAME: Fults, R.D., DAC NAME: Kretsch, M.J., DAC POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Nutrition and Environment; (U) Body Composition; (U) Nutrient Requirements; (U) Nutrition and Performance; (U) Human Volunteers							
23. (U) TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Attainment and maintenance of optimum metabolism is of prime import to the Armed Forces and requires a constant search for new methods for improving and evaluating physiological status. Status reflects nutriture and body hydration, and may be affected by age, sex, state of health, dietary constituents, vitamins, hormones, therapeutic agents, and varied stresses, all of which should be reflected in body composition changes and related physiological parameters. General objective is to conduct fundamental research that will provide guidance about dietary alterations and nutritional practices that can be applied for increased efficiency of military personnel operating under various combat and environmental stresses. 24. (U) Approaches will be to (a) investigate the physiological basis of nutrition, pulmonary function, and physical status, utilizing human subjects and experimental animals; (b) study the physiologic and metabolic adaptations to nutritional and environmental stresses, including research on the relationship of nutrient requirements; and (c) develop techniques and biomedical instrumentation to measure body composition, caloric requirements, and other nutrient requirements; and (d) investigate through the use of animal models the metabolic and/or neurophysiological basis of stress and diet-induced changes in voluntary food and water consumption. 25. (U) 76 10 - 77 09 Computer programming for evaluating and improving body composition measurements and feedback control (from pulse rates, oxygen consumption, etc.) of treadmill operations are being completed. Respiratory quadrupole mass spectrometer has been built and is being tested by contract. Protocols utilizing these new techniques for investigating nutritional requirements for military personnel and effects upon military performance are being drafted. Data for two human protein studies on requirements during physical training are being computer-processed and evaluated.							

# ABSTRACT

PROJECT NO.	3M161102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	057	Nutritional Physiology in Health and Prevention of Injury in the Military

The following investigations have been conducted under this work unit:

STUDY NO. 6 Evaluation of protein requirements during heavy physical activity

STUDY NO. 7 Development of instrumentation and methodology for evaluating physiological status

STUDY NO. 6. Analyses of samples and processing of data collected from two studies on the adequacy of protein allowances during heavy physical training (e.g., basic training of military inductees) have been ongoing. Concentrations of nitrogen, sodium, potassium, calcium, and magnesium were determined from samples of blood, feces, sweat, and urine. Work performance and body compositional data are being processed as computer programming is being completed, and punched paper tape problems are being resolved. Most of the data are now ready for statistical analyses and evaluations. Preliminary indications were that NRC allowances of protein (0.6 g/kg of body weight) would maintain nitrogen balances after adapting to the diet, while military allowances (1.4 g/kg of body weight) were adequate for protein or muscle synthesis during physical training. Further evaluations will be made after analyzing the work performance and body compositional data.

STUDY NO. 7. The acquisition of the minicomputer for on-line data processing and experimental control and the quadropole mass spectrometer (under construction by contract) will greatly enhance our capabilities for determining nutrient requirements of personnel undergoing stresses commonly encountered in military situations and for evaluation of the physical and physiological status of military personnel. However, these acquisitions have also created the need for instrument interfacing and computer programming prior to their use. Most of the updating of equipment and techniques to the current state-of-the-art have progressed expeditiously and is nearing completion. Specific protocols with the use of these techniques are being developed to evaluate nutrient requirements of personnel performing military duties in different environments.

## BODY OF REPORT

WORK UNIT NO. 057

Nutritional Physiology in Health and Prevention of Injury

STUDY NO. 6

Evaluation of protein requirements during physical training and heavy physical activity

### PROBLEM

Basic training for military inductees, advanced and specialized training for most combat arms personnel, and the refresher training upon activation of the National Guard or Reserve personnel have a large portion of time dedicated to physical training and conditioning. Any factors that would contribute to the efficiency and effectiveness of these programs would benefit all services and the personnel undergoing training. The amounts of dietary protein required during physical conditioning have been a long-term controversy (for at least 120 years) and have recently reappeared in the literature. This study was designed to evaluate the adequacy of the National Academy of Sciences-National Research Council's Recommended Allowances (NRC) versus those of the military (AR 40-25) during periods of physical conditioning and heavy physical activity.

### RESULTS AND DISCUSSION OF RESULTS

The first two phases of this study were conducted during January through April 1976, and the experimental details were presented in the Annual Report FY 76. Mineral and nitrogen catabolite concentrations were determined in the samples of blood, urine, feces, and sweat collected during the study. Oxygen consumption and other work performance data from the treadmill tests have been extracted and are undergoing evaluation. Programming of the body composition and pulmonary function data reduction are nearing completion, and these data will be processed in the near future. Preliminary information from nitrogen balance data indicated that balances could be attained at the NRC allowance of protein (0.6 g/kg of body weight) during physical conditioning after a 12-day sedentary period of adaptation to the low dietary levels of protein, and that the military allowances (100 g/day or 1.4 g/kg of body weight) were adequate for nitrogen retention, indicative of increased muscle mass or body protein stores. Positive potassium balances were consistent with these results. These results were obtained by using well-defined highly digestible liquid diets.

### CONCLUSIONS

Initial indications were that the NRC allowances for protein would provide for nitrogen balances during physical training after adapting to these diets, and that military allowances would provide sufficient



protein for increasing muscle mass. The effects of these protein levels upon body composition and work capacities will be assessed when data reduction and analyses have been completed.

#### RECOMMENDATIONS

Data analyses should be completed and the final phase of the study should be conducted, probably with the use of normal military diets. The design for the final phase of the study will depend upon the complete evaluation of the first two phases.

#### PUBLICATIONS

1. JOHNSON, H.L., C.F. CONSOLAZIO, D.D. SCHNAKENBERG, M. GUHMAN, and B.W. SCHWENNEKER. The effects of initiating heavy physical training of man upon mineral excretions and balances. (Abstract) Fed Proc 36: 1130, 1977.
2. CONSOLAZIO, C.F., D.D. SCHNAKENBERG, H.L. JOHNSON, J. SKALA, R. FULTS, and S. HULL. Evaluation of the NRC protein allowances during heavy physical activity (Abstract) Fed Proc 36: 1172, 1977.
3. CONSOLAZIO, C.F., and D.D. SCHNAKENBERG. Nutrition and the responses to extreme environments. Fed Proc 36: 1673, 1977.

STUDY NO. 7

Development of instrumentation and methodology for evaluating physiological status

#### PROBLEM

The effects of nutritional and environmental stresses are reflected by physiological status, including cardiopulmonary function, body composition, and physical work capacity. Highly sophisticated instrumentation and methodology are required to investigate adequately the effects of stress on these parameters. In addition, many military situations require the soldier to perform strenuous physical activities while under nutritional and environmental stress. To ensure that the soldier is able to perform adequately under such conditions, heavy physical activity must be incorporated into experimental protocols. Since physical activity increases the difficulty of measuring physiological parameters, more complex instrumentation is required for studies involving activity than for studies under static conditions. Complex instrumentation and methodology also allow investigation of the dynamics of the responses to stress, an important factor in understanding the response. The development of a laboratory incorporating high-speed instrumentation, automated data acquisition, complex computerized analysis procedures, and suitable methodology will facilitate extensive evaluation of the effects of nutritional and environmental stress.



## RESULTS AND DISCUSSION OF RESULTS

All developmental work is aimed at automating data acquisition under control of the Modcomp II minicomputer (MCII) and providing data processing on the MCII, the Data General Eclipse (C3000) computer in the Department of Information Sciences, or the CDC 7600 system at Lawrence Berkeley Laboratories (LBL).

The treadmill automation system (TAS), a computer software package for controlling real time computer data acquisition by the MCII during treadmill experiments, was delivered by LBL in July 1977. Preliminary tests for software in the LAIR environment indicate that it will perform as intended. Data acquisition from the continuous gas analyzer is currently being implemented to operate under TAS on the MCII.

The digital treadmill controller, providing stand-alone program control of treadmill speed and elevation as well as an interface to the MCII for program control under TAS, has undergone final testing. Its control functions are presently being integrated with TAS. Development of a similar controller for the laboratory's bicycle ergometers has been initiated.

The Small Animal Feeding Data Acquisition System (SAFDAS) has passed final engineering tests and is ready for laboratory use. Hardware and software development to facilitate data transfer from the Datel digital cassette recorder to the MCII has been completed. This is a prerequisite for computer processing of data collected via SAFDAS. In preparation for the delivery and installation (second quarter FY 78) of the Respiratory Mass Spectrometer (RMS) being developed under contract at the University of Colorado, a Data General NOVA 1220 minicomputer was acquired from excess property. Hardware and software modifications were ordered to facilitate RMS control via the NOVA 1220. Assembly of the interface between the RMS and NOVA 1220 is in progress.

Software has been developed on the MCII which provides a data line, via acoustic coupler and telephone line, from the MCII to the Remote Equipment Control System at LBL. Via this data line, data acquired with the MCII will be transferred to the CDC 7600 computer system at LBL where extensive manipulation and statistical analyses are possible.

Software is under development to allow integration of the MCII into the LAIR minicomputer network managed under the C3000. Methods have been developed to format data strings acceptable to the C3000 and append cyclic redundancy code check words to the strings, enabling transmission error detection.

A heart-rate monitor interface for the MCII has been designed and assembled. It is now being optimized. The interface will allow the MCII, operating under TAS control, to monitor heart rate on a beat-by-beat basis during exercise studies.

The interface for the data line connecting the K40 shadow shield counter to the MCII has been designed. Assembly will proceed when components, which are on order, have been received. Software for managing K40 data filed on the MCII is 80% completed. Software for extended analysis of K40 data is under development and is 25% completed.

The acquisition of the minicomputer for on-line data processing and experimental control and acquisition of the quadrapole mass spectrometer (under construction by contract) will greatly enhance our capabilities for determining nutrient requirements for personnel undergoing stresses commonly encountered in military situations and for evaluation of the physical and physiological status of military personnel. However, these acquisitions have also created the need for instrument interfacing and computer programming prior to their use. Most of this updating of equipment and techniques to the current state of the art has progressed expeditiously and is nearing completion. Specific protocols in which these techniques are used are being developed to evaluate nutrient requirements of personnel performing military duties in different environments.

#### PUBLICATIONS

BURK, R.F., H.E. JORDAN, JR., and K.W. KIKER. Some effects of selenium status on inorganic mercury metabolism in the rat. *Toxicol Appl Pharmacol* 40:71, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6114	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	61102A	3M161102BS02		00		061	
b. <del>ORIGINATOR</del>							
c. <del>ORIGINATOR</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Disease Mechanisms at the Cellular Level							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology; 010100 Microbiology; 002300 Biochemistry; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-house	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
d. NUMBER <sup>a</sup>				77		1.5	
c. TYPE: Not Applicable				FISCAL YEAR		88	
e. KIND OF AWARD:				78		1.7	
f. CUM. AMT.						147	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> Bucci, T.J., LTC, VC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-4019			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Histochemistry; (U) Electron Microscopy; (U) Diagnosis; (U) Infection; (U) Laboratory Animal; (U) Metabolic Disease							
23. (U) Prevention or control of disease depends on complete understanding of the abnormal processes involved, from initial injury of the cell to repair. These experiments will encompass diseases of military importance such as skeletal injury of soldiers and economically important diseases of laboratory animals. They will provide information on cellular response to specific injury and will form a basis for studies of a more clinical nature.							
24. (U) Diseases of particular importance to the Army which are poorly understood will be studied at the cellular level in appropriate human or animal cells. Electron microscopy, histochemistry, and quantitative analytical methods will be used. Basic differences between normal and affected cells will be sought, and hypotheses regarding the disease mechanism will be proposed and tested. Initial studies will involve control of calcium metabolism and early detection of diseases of laboratory animals. A new enzyme histological technique, peroxidase-antiperoxidase localization of biologically important proteins, will be standardized and implemented.							
25. (U) 7610-7709 Progress has been hampered by shortage of trained technicians. Feline syncytial virus (FSV) and owl monkey adenovirus (OMA), both prevalent infections, have been chosen as test systems. Antigens have been purified and antibodies are being prepared to attempt rapid, specific diagnosis of these and subsequently other viral diseases of laboratory animals, precluding the need for virus isolation. The technique will also permit study of the pathogenesis of troublesome diseases (e.g., FSV and OMA), thus contributing to eventual control of those diseases. Localization of calcium-binding protein in studies of human bone disease are in progress.							



# ABSTRACT

PROJECT NO.	3M161102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	061	Disease Mechanisms at the Cellular Level

The following investigations have been conducted under this work unit:

STUDY NO. 1      Feasibility of the use of immunoenzyme-labeled  
antibody in the histopathological diagnosis of  
viral laboratory animal diseases

STUDY NO. 2      Role of CaBP in mineral metabolism

NON-NUMBERED STUDIES (reported under work units of other LAIR  
investigators)

Work performed under this work unit included animal necropsy, histopathology, clinical pathology (hematology, clinical chemistry, microbiology), scanning and transmission electron microscopy, and special morphometric procedures. These techniques were applied to two numbered studies addressing cellular responses in disease, and to 30 additional studies in collaboration with or in support of the principal investigators of the additional studies. The latter effort represented complete support in veterinary pathology for all LAIR and LAMC investigators who required it. The specific work units and programs supported are tabulated in the text of this report, and all resulting publications are listed.

STUDY NO. 1. This study was delayed until a technician was obtained in January 1977, and subsequently trained. Accomplishment was principally in preparation and testing of specific antisera and antiserum-enzyme conjugates to be used in diagnostic studies of laboratory animal diseases. These reagents and candidate tissues are now available, and tests are in progress to evaluate the feasibility of immunohistochemistry for rapid detection of feline herpesvirus and owl monkey adenovirus infection.

STUDY NO. 2. Independent investigations in this study were precluded by departure of a co-investigator and both experienced technicians. An untrained replacement technician became available for 8 months. His on-the-job training was accomplished through his performance of related collaborative studies under supervision of the remaining part-time investigator. The conclusions resulting from this work are reported under Project No. 3A161101A91C, Work Unit 060, Study No. 9; localization of calcium-binding protein for those experiments were performed under this work unit.



NON-NUMBERED STUDIES. Work accomplished in support of, or in collaboration with, other LAIR investigators is reported under their work units, as tabulated in the body of this report. Results of investigations supported for Clinical Investigation Service, LAMC, are described in the LAMC report. One study which was begun as a collaborative effort with LAMC and subsequently discontinued by them was converted to an investigation of gastrointestinal transit time and completed under this work unit. The work was done in rhesus monkeys, and the conclusion was that transit time of a marker meal (barium labeled, monitored radiographically) could not distinguish normal animals from those with a history of (and therefore future predisposition towards) acute gastric dilatation.

## BODY OF REPORT

WORK UNIT NO.	061	Disease Mechanisms at the Cellular Level
STUDY NO.	1	Feasibility of the use of immunoenzyme-labeled antibody in the histopathological diagnosis of viral laboratory animal diseases

### PROBLEM

Healthy laboratory animals are a vital resource for Army medical research. Diseased animals are wasteful of this resource and the loss is compounded when experiments cannot be evaluated properly because of intercurrent disease in the research animals. Accurate and expeditious means to detect and diagnose diseases, particularly viral diseases, are therefore highly desirable. It is usually impractical to use standard procedures to culture, isolate, and identify the virus(es) that cause disease in laboratory animals. Techniques using enzyme-labeled specific antibody have been used for rapid detection and identification of viral antigens in human tissue. The advantage of these techniques lies in their rapidity, sensitivity, and reliability. This method will be adapted to detect and identify viral antigens in fixed and frozen tissue as a rapid and economical means to diagnose specific viral diseases of laboratory animals. When the technique is established for a model system, it will be adapted by using appropriate antiserum for quick identification of other specific viral antigens which may be present in the tissues of both sick and apparently healthy laboratory animals.

Candidate diseases for which a rapid specific detection method is needed include herpes and syncytial viral (FeSFV) infection in cats, adenoviral infection in owl monkeys, and Sendai viral infection in rodents. Surveillance for presence of viral agents is conducted under Project No. 3M762772A812, Work Unit No. 002, Study No. 3; viral isolates and known infected tissue from that work are effectively used in this study. If this study is successful, this relatively inexpensive technique will preclude future need for expensive culture, isolation, and identification of these and similar viruses.

### RESULTS AND DISCUSSION OF RESULTS

We chose feline herpesvirus as the model to test the feasibility of enzyme-labeled antibody for immunohistochemical diagnosis. This virus was chosen for these reasons: (1) cats are common experimental animals at LAIR, and this virus causes a wide-spread and significant disease in laboratory cats; (2) the virus grows readily in vitro (the disease and the virus are both well-studied); and (3) disease caused by

a herpesvirus is an appropriate model because most laboratory species are infected by at least one virus of this group.

In January 1977, a technician became available for training in the enzyme-labeled antibody technique. The method requires specific antiserum (which we prepare in rabbits), specific antigen (e.g., feline herpesvirus), and a conjugate composed of antibody to rabbit gamma globulin and the enzyme peroxidase. This latter antibody can be prepared in any species (e.g., sheep or goat) and is also available commercially.

Tissues were collected from cats used in another study. All had been in the LAIR colony for at least 6 months. During this time pharyngeal swab samples were collected periodically for isolation of viruses and stored for eventual correlation with the enzyme techniques. Tissue from infected and non-infected animals was collected at necropsy and stored for subsequent staining with enzyme-labeled antiserum. Cell cultures of feline renal cells infected with feline herpesvirus were prepared as positive controls for the test and are currently being used to standardize the reagents.

Rabbit antisera are being prepared against gamma globulin and adenovirus of owl monkeys and against FeSFV. These reagents and the standardized technique will permit diagnosis and evaluation of the full implication to the animal of infection with these agents.

#### CONCLUSIONS

Development of a peroxidase-labeled antibody method to diagnose viral diseases in laboratory animals appears feasible.

#### RECOMMENDATIONS

Standardization of prototype systems for diagnosis of viral diseases of laboratory animals with the use of peroxidase-labeled antibody should be completed. Antisera to numerous diseases should be evaluated for diagnostic potential.

#### PUBLICATIONS

None

STUDY NO.	2	Role of CaBP in mineral metabolism
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#### PROBLEM

Many aspects of the role of vitamin-D dependent, calcium-binding protein (CaBP) can be inferred from its location within or on cells of laboratory animals under various defined physiologic conditions. This study was designed to examine these aspects and contribute to an

understanding of mineral metabolism and to prevention or improved management of skeletal injuries in military personnel.

Progress was markedly hampered by delayed replacement of technicians. In the prior year, work was performed by 2 part-time investigators and 2 full-time, fully trained and experienced technicians. Only one part-time investigator remained after August 1976, and one inexperienced and untrained technician became available in January 1977. Most of his training was accomplished while he performed CaBP localizations in collaborative investigation on a related study (Project No. 91C, Work Unit No. 060, Study No. 9). The CaBP localizations performed for the collaborative experiments were funded by this work unit and resulted in joint publications.

#### RESULTS AND DISCUSSION OF RESULTS

The major independent achievement was development of a trained technician who is now competent to perform chromatographic separation of antibodies from serum, perform the range of tissue processing from frozen sectioning to preparation of 0.5 micrometer plastic-embedded sections, and perform several variations of the peroxidase-labeled antibody localization of tissue antigens. One study unfinished from the previous year required photography of CaBP localized in rat fetuses and established that the protein becomes demonstrable for the first time at the 19th gestational day (two days before birth). The sequence of intranuclear CaBP versus intracytoplasmic CaBP added support to previous results, strengthening the hypothesis that CaBP affects gene expression in certain cell populations.

#### CONCLUSIONS

Progress was hampered by lack of technical help. Sufficient work was accomplished to contribute to the conclusions expressed for Project No. 91C, Work Unit No. 060, Study No. 9.

#### RECOMMENDATIONS

The technician's experience and training should progress to enable him to perform the technique on specimens suitable for electron microscopic analysis. Study No. 2 will be terminated.

#### PUBLICATIONS

Note: Asterisk (\*) designates the author(s) on the staff of the Department of Comparative Medicine.

1. MORRISSEY, R.L., R.M. COHN, R.N. EMPSON, JR.\*, H.L. GREEN, O.D. TAUNTON, and Z.Z. ZIPORIN. Relative toxicity and metabolic effects of cholecalciferol and 25-dihydroxycholecalciferol. J Nutr 107:1027-1034, 1977



2. MORRISSEY, R.L., D.T. ZOLOCK, D.D. BICKLE, R.N. EMPSON, JR.\*, and T.J. BUCCI\*. Intestinal response to  $1\alpha,25$ -dihydroxycholecalciferol: I. RNA polymerase, alkaline phosphatase, calcium and phosphorus uptake in vitro and in vivo calcium transport and accumulation. *Biochim Biophys Acta* 538:23-33, 1978
3. MORRISSEY, R.L., R.N. EMPSON, JR.\*, D.T. ZOLOCK, D.D. BIKLE, and T.J. BUCCI\*. Intestinal response to  $1\alpha,25$ -dihydroxycholecalciferol: II. Cellular localization of calcium binding protein. *Biochim Biophys Acta* 538:34-41, 1978
4. BIKLE, D.D., R.N. EMPSON, JR.\*, R.L. MORRISSEY, D.T. ZOLOCK, R.H. HERMAN, and M.M. PECHET. Sequential changes in the rachitic chick following  $1\alpha,25$  OH  $D_3$  treatment. *Endocrinology* 98:258, 1976
5. EMPSON\*, R.N. JR., T.J. BUCCI\*, R.L. MORRISSEY, and J.S. CHANDLER. Restriction of renal lesions induced by vitamin  $D_3$  to tubules containing calcium binding protein. *Am J Pathol* 82:55-56, 1976
6. MORRISSEY, R.L., D.T. ZOLOCK, T.J. BUCCI\*, and D.D. BIKLE. Immunoperoxidase localization of vitamin D dependent calcium binding protein. *J Histochem Cytochem* (in press)
7. MORRISSEY, R.L., D.T. ZOLOCK, D.D. BIKLE, and P.W. MELLICK\*. Role of vitamin-D dependent calcium binding protein in intestinal calcium absorption. (Abstract) *Fed Proc* 36:1097, 1977
8. BIKLE, D.D., R.N. EMPSON, JR.\*, R.L. MORRISSEY, D.T. ZOLOCK, T.J. BUCCI\*, R.H. HERMAN, and M.M. PECHET. The action of  $1\alpha,25$  hydroxy vitamin  $D_3$  on rachitic chick intestine: Rate of response of RNA polymerase, calcium binding protein, and alkaline phosphatase. (submitted for publication)
9. BIKLE, D.D., R.N. EMPSON, JR.\*, R.H. HERMAN, R.L. MORRISSEY, and D.T. ZOLOCK. The effect of  $1,25$  dihydroxyvitamin  $D_3$  on the distribution of alkaline phosphatase activity along the chick intestinal villus. *Biochim Biophys Acta* 449:61-66, 1977

#### NON-NUMBERED STUDIES

In addition to the cell-level studies of disease mechanisms, this work unit provided support in pathology and electron microscopy for all investigators of LAIR and Clinical Investigation Service (CIS), LAMC. Specific veterinary pathologic services, i.e., animal necropsy, microbiology, histopathology, and clinical pathology; and special techniques, i.e., histochemistry, morphometry, and electron microscopic autoradiography, were performed. Based on careful records for January through September 1977, approximately \$32,000 was spent from this work unit for pathologic services to investigators in other work units. The sum includes civilian technicians' pay and benefits, expendable supplies,

and prorated overhead for civilian and military technicians. Officer (investigator) salaries and overhead are not included in this figure.

Support and collaboration by veterinary pathologists were provided for the LAIR studies listed below. Outcome of those studies is reported elsewhere in this annual report. Publications resulting from collaborative efforts are listed at the end of this section.

LAIR STUDIES PROVIDED WITH SUPPORT/COLLABORATION  
BY VETERINARY PATHOLOGISTS

<u>Project No.</u>	<u>Work Unit</u>	<u>Project No.</u>	<u>Work Unit</u>
3A161101A91C	042	3M762772A811	002
	043	3M762772A812	001
	045		002
	051		003
	060		024
3M161102BS02	056	3E762772A813	025
	065	3S762772A814	001
	067		004
	071		008
	075		011
	062		012
3M762772A810	001		014

Support and collaboration was provided by veterinary pathologists to CIS, LAMC, for several studies, the majority of which are still incomplete. The services include animal tissue evaluations in studies of synthetic prostheses for large veins, treatment of experimental spinal cord trauma, laser hemostasis in urinary bladder, induced pregnancy toxemia, and penetrating renal trauma.

A collaborative research project with CIS, LAMC, was to have been an evaluation of anterior gastropexy for treatment of hiatal hernia. The first phase was to establish normal gastrointestinal motility; then, the next phase was to perform the gastropexy and measure its effect on motility. Six M. mulatta monkeys were used in a part of the project which was completed, i.e., the conditioning of the animals to a restraint

board. The research was discontinued because the LAMC investigator departed without a replacement. Coincidentally, 2 of the 6 M. mulatta monkeys conditioned for the gastropexy/hiatal hernia study had histories of acute gastric dilatation (bloat). To capitalize on the invested effort and the training achieved, we chose to use the animals to compare transit time in bloaters versus non-bloaters.

Acute gastric dilatation (bloat) is a sporadic and serious condition in nonhuman primates. The gastrointestinal emptying time for normal versus bloater monkeys was unknown. If there are differences in gastrointestinal transit times between bloaters and non-bloaters, emptying time could be used as the criterion to identify potential bloaters. Non-bloater monkeys could be selected for studies, and monkeys that are potential bloaters could be watched more carefully so that early treatment could be instituted.

Gastrointestinal transit time for liquid and semisolid meals was measured with radiographic contrast procedures in the six adolescent monkeys. The effect of anesthesia on transit time was also determined.

The liquid meal consisted of barium sulfate, 28% w/w in water (20 ml/kg) and the semisolid meal was made by adding 10 gm of mashed banana per ml of barium suspension. Meals were delivered by stomach tube. Movement of contrast medium through the stomach and small intestine and its entrance into the cecum were recorded at 15 minute intervals. The procedure was repeated with each animal three times at weekly intervals. Animals were awake for the first two procedures and anesthetized with ketamine HCl (intramuscular injection, 10 mg/kg initial; 5 mg/kg maintenance) before administration of the third. The monkeys were preconditioned for restraint on an immobilization device.

Barium was in the duodenum within 15 minutes in all normal and bloater monkeys regardless of anesthesia or consistency of the test meal. The following table is a tabulation of the time in minutes for transit of the barium marker.

#### GASTROINTESTINAL TRANSIT TIME OF A BARIUM MEAL IN RHESUS MONKEYS

<u>Meal</u>	<u>Monkey</u>	<u>Transit Time in Minutes</u>	
		<u>Small Intestine</u>	<u>Enter Cecum</u>
Liquid	Unanesthetized		
	Normal	34	45
	Bloaters	33	45
Semisolid	Normal	71	87
	Bloaters	48	48
Semisolid	Anesthetized		
	Normal	105	133
	Bloaters	50	54



In normal monkeys, semisolids were transported more slowly than liquids and ketamine anesthesia delayed intestinal transit of semisolids. In bloater monkeys, there was little difference in transport time between liquids and semisolids and the time was unaffected by ketamine anesthesia.

A second study was done to expand these observations and to verify the apparent difference in intestinal transport time between bloater and non-bloater monkeys. Nine rhesus monkeys, 2 to 5 years of age, were selected. The investigator remained uninformed as to which 3 had a history of bloat. Preparation and administration of the semisolid meal, induction and maintenance of anesthesia, and radiographic procedures were the same as described above. One trial per animal was performed.

The second study showed that bloater monkeys could not be identified on the basis of their intestinal transport time. There were marked individual differences in transit times within both groups. It was concluded that intestinal transport time is an unreliable indicator of a history of acute gastric dilatation in rhesus monkeys.

ADDITIONAL PUBLICATIONS AUTHORED/CO-AUTHORED BY STAFF OF THE DEPARTMENT OF COMPARATIVE MEDICINE

Note: Asterisk (\*) designates the author(s) on the staff of the Department of Comparative Medicine.

1. BEATRICE, E.S., D. TALSMA, and S. SCHUSCHERBA\*. Retina: Electron microscopy one and three years after laser exposure. (Abstract) Invest Ophthal Visual Sci, Supplement, 1977. p 18
2. FRIEDMAN, H.E., F. DEVENUTO, T.F. ZUCK, P.W. MELLICK\*, and L.O. LOLLINI\*. The histological and ultrastructural effects of stroma-free hemoglobin solutions on rat liver, kidney, and brain. Surgical Forum 28:3-5, 1977
3. HANNON, J.P\*. Comparative altitude adaptability of young men and women. In: Environmental Stress: Individual Adaptations, Proceedings of International Symposium (Santa Barbara, CA, 31 Aug - 3 Sep 1977), edited by J.J. Folinsbee. New York: Academic Press (in press)
4. GESSAMAN, J.A., T. ADAMS, J.P. HANNON\*, C. JONKEL, and R.G. WHITE. Energy and nutrient acquisition and utilization. In: Symposium on Comparative Mechanisms of Cold Adaptations in the Arctic (Michigan State University, East Lansing, MI, 24-25 Aug 77), edited by L.S. Underwood. Arlington, VA: Am Inst of Biol Sci, 1977. p 5
5. HANNON\*, J.P., and J.A. VOGEL. Oxygen transport during early altitude acclimatization: A perspective study. Europ J Appl Physiol 36:285-297, 1977



6. McANINCH, J.W., W.G. RODKEY, R.E. STUTZMAN, and T.J. BUCCI\*.  
A mechanical device for standardization of experimental penetrating renal injury. (submitted for publication)
7. SHROYER, E.L\*. Food as a vehicle for virus transmission.  
(Abstract) J Am Vet Med Ass 171:1099, 1977
8. WARD, G.S\*., D.C. JOHNSEN\*, R.M. KOVATCH\*, and T. PEACE\*.  
Myopathy of guinea pigs. J Am Vet Med Ass 171:837-838, 1977
9. PLOPPER, C.G\*., D.L. WALLACE\*, T.J. BUCCI\*, and H.E. SAUBERLICH.  
Autoradiographic localization of vitamin A in the kidney of rats.  
Proc Soc Exp Biol Med 155:124-127, 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. ORIGIN INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
77 07 19	D. CHANGE	U	U	NA	NL		
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61102A	3M161102BS02		00	062		
b. CONTRIBUTING							
c. <del>XXXXXXXXXX</del>	CARDS 114 f						
11. TITLE (precede with Security Classification Code) <sup>a</sup> (U) Response of the Respiratory System to Injury							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002600 Biology; 012900 Physiology; 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER:				FISCAL		1.0	
c. TYPE: Not Applicable				YEAR		78	
d. AMOUNT:				CURRENT		48	
e. KIND OF AWARD:				78		.7	
f. CUM. AMT.						89	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
ADDRESS: Presidio of San Francisco, CA 94129				ADDRESS: Pathology & Comparative Studies Div			
				Department of Comparative Medicine			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME: Mellick, P.W., MAJ, VC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-3855			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Respiratory Injury; (U) Cell Kinetics; (U) Wound Repair; (U) Electron Microscopy; (U) Airway; (U) Chemical Irritants; (U) Smokes							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Upper respiratory infection is the most common illness that affects military personnel. Soldiers are frequently exposed occupationally to airborne chemical agents that damage respiratory tissues. Knowledge of basic mechanisms by which respiratory epithelium is repaired following injury is inadequate and is required so that clinical management of personnel with acute respiratory injury can be improved. An animal model to study basic repair mechanisms in the respiratory tract will be developed. 24. (U) The initial experiment in this work will be done in rats. Animals will be exposed to sublethal concentrations of a corrosive gas to produce respiratory injury. The duration of cell cycle phases in exposed and control animals will be determined at three levels of the respiratory tract by autoradiographic techniques. Epithelial proliferation and differentiation will be studied by light microscopy, scanning and transmission electron microscopy, and electron microscopic autoradiography. Semiquantitative techniques to assess the regenerative capacity of respiratory epithelium will be developed and standardized. Smokes and gases noxious to troops can then be compared. 25. (U) 7610-7709 Thirty-two rats were exposed to sublethal concentrations of ozone to produce injury to respiratory epithelium. A control group of 32 rats were exposed to filtered air. Rats were injected with tritiated thymidine immediately following exposure and killed at intervals to determine cell turnover time and length of component phases of the cell cycle in control and injured rats. Trachea and lung tissues from all animals were preserved for histologic and ultrastructural evaluation. Tissues from 44 rats have been processed for light microscopic autoradiography. Tissues from 10 animals killed at different times following exposure have been examined by transmission and scanning electron microscopy to evaluate the degree of damage and to document repair processes. A capability to do electron microscopic autoradiography has been developed and evaluated. Tissues from 6 animals have been studied by this process.							

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

# ABSTRACT

PROJECT NO.	3M161102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	062	Response of the Respiratory System to Injury

The following investigation has been conducted under this work unit:

STUDY NO. 1 Epithelial regeneration in the upper respiratory tract following acute oxidant injury

A group of 32 weanling rats were exposed to a sublethal concentration (0.8 ppm) of ozone for 8 h. Following exposure these animals and a group of 32 control rats were given 500  $\mu\text{Ci}$   $^3\text{H}$  thymidine by intraperitoneal injection. Two exposed and two control rats were killed at intervals of 1/2, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 14 h. One rat of each group was killed 24, 48, 72, 96, 120, 144, and 168 h after injection of the isotope. Lungs and tracheas of all animals were preserved for examination by light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and LM and TEM autoradiography. Tissues from the 48 animals killed during the first 14 h have been examined by LM and prepared for LM autoradiography. Analysis of LM autoradiography preparation from these animals to determine the duration of component phases of the cell cycle is in progress. Examination of tissues from 10 animals by TEM and SEM indicates that the degree of damage produced in terminal bronchioles should be sufficient to stimulate repair processes. A capability to do autoradiography at the ultrastructural level has been developed and is being evaluated. This technique will be used to study cell differentiation and migration in airway epithelium of ozone-exposed and control rats.

## BODY OF REPORT

WORK UNIT NO. 062

Response of the Respiratory System  
to Injury

STUDY NO. 1

Epithelial regeneration in the upper  
respiratory tract following acute  
oxidant injury

### PROBLEM

Upper respiratory infection is the most common illness that affects military personnel both in training and in combat situations. Soldiers are frequently exposed occupationally to airborne chemical agents that damage respiratory tissues. Of the 91 chemical compounds that are components, by-products, or residues of pyrotechnic devices in use or under development by DOD agencies, 25 have been shown to be moderately or severely toxic to humans or animals when they inhale the fumes. Explosive and incendiary munitions have similar chemical composition and, therefore, constitute an additional toxic hazard especially to troops exposed in confined spaces such as bunkers, entrenchments, and armored vehicles. Epidemiological evidence and clinical experience indicate that chemically induced injury to respiratory tissues can predispose a person to upper respiratory infection and other non-infectious but equally debilitating pulmonary disease.

Knowledge of basic mechanisms by which upper respiratory epithelium heals is inadequate and is required so that treatment and clinical management of patients with upper respiratory injury can be improved. This study was initiated to develop a laboratory animal model for study of basic repair mechanisms in the respiratory tract.

### RESULTS AND DISCUSSION OF RESULTS

Upper respiratory epithelial injury was produced in rats by subjecting them to a non-lethal concentration (0.8 ppm) of a highly corrosive gas (ozone) for 8 h. Exposed and control rats were given 500  $\mu\text{Ci}$   $^3\text{H}$  thymidine by intraperitoneal injection immediately following ozone exposure. Rats were killed at intervals following injection of the isotope. Trachea and lungs from each animal were fixed by airway perfusion with Karnovsky's fixative for subsequent examination by light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and LM and TEM autoradiography.

No lesions were detected in tissues of control animals. Examination of tissues from selected exposed rats by LM and SEM indicated that the degree of epithelial injury produced by ozone exposure should be sufficient to stimulate epithelial regeneration in terminal bronchioles and alveolar ducts. Further evaluation of tracheas is necessary to determine whether or not sufficient damage was produced. The rat lacks



respiratory bronchioles which are well-developed in human lungs; and so evaluation of this segment of conducting airway is not possible in rats.

Cross sections of mid-cervical trachea and longitudinally bisected sections of terminal bronchioles from the 48 rats killed during the first 14 h post injection have been embedded in Epon-Araldite, sectioned at a thickness of 1  $\mu$ m and coated with photographic emulsion for LM autoradiography. LM autoradiographic preparation of 104 of these 152 tissue specimens is complete and the remaining 48 specimens are in progress. Preliminary examination indicates that the LM autoradiographic preparations are of sufficient quality to permit cell cycle analysis. Evaluation of these 152 specimens will permit a comparison of cell cycle times and duration of component phases in control and exposed rats.

Tissues from 7 control and 7 ozone-exposed animals (one of each group killed at 24 h intervals through 7 days post exposure) will be processed for LM and TEM autoradiography. Study of these tissues will document cell migration and differentiation and should reveal the identity of precursor cells in trachea and terminal bronchioles. Preliminary results of TEM autoradiography of this material were unsatisfactory because of defective emulsion, inadequate exposure time, improper coating technique, and lack of precision in sampling. These problems have been overcome, and recent preparations are satisfactory. Initial progress in this study was hampered by lack of sufficient technical personnel and our commitments to support other studies. The acquisition of two skilled technicians during this year has alleviated this problem to some degree, and current progress is satisfactory.

#### CONCLUSIONS

Exposure of rats to 0.8 ppm ozone for 8 h produces sufficient damage to the upper respiratory epithelium to stimulate epithelial regeneration. This model has great value in that it is relatively inexpensive and should provide insight into the basic mechanisms of airway epithelial repair. The value of the rat as an animal model of human pulmonary disease is limited because this species lacks well developed respiratory bronchioles which are the prime targets for inhaled noxious chemical agents in man.

#### RECOMMENDATIONS

This study should be continued to completion. Other studies should be instituted to identify a species of animal that has distal airway morphology more closely resembling that of man. Then with a more appropriate animal model, definitive studies of the response of the respiratory system to injury should be pursued.

#### PUBLICATIONS

1. MELLICK, P.W., D.L. DUNGWORTH, L.W. SCHWARTZ, and W.S. TYLER.  
Short term morphologic effects of high ambient levels of ozone on  
lungs of rhesus monkeys. Lab Invest 36:82-90, 1977
2. TYLER, W.S., L.W. SCHWARTZ, D.L. DUNGWORTH, P.W. MELLICK, and L.A.  
ZITMIK. Nonciliated bronchiolar epithelial (Clara) cell response to  
injury. In: Lung Cells in Disease, Proceedings of a Brook Lodge  
Conference (Augusta, MI, 21-23 April 1976), edited by A. Bouhuys.  
New York: North-Holland Biomedical Press, 1976. pp 179-180

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6102	77 10 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISB'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
	61102A	3M161202BS02		00	065		
B. CONTRIBUTING							
C. CONTINUING		CARDS 114f					
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Military Stress and Combat Effectiveness							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
005900 Environmental Biology; 013400 Psychology; 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 08		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES, EFFECTIVE:		EXPIRATION:		PRECEDING		B. FUNDS (In thousands)	
B. NUMBER <sup>a</sup>				FISCAL YEAR		77 7.5 152	
C. TYPE: Not Applicable		D. AMOUNT:		CURRENT		73 7 140	
E. KIND OF AWARD:		F. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
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				NAME: Stamper, D. A., DAC			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup> (U) Military Performance; (U) Psychological Stress; (U) Stress and Disease; (U) Primate Bloat							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The severe stress encountered in warfare may influence the soldier's ability to perform combat essential activities with maximum efficiency. The objectives of this research are to study, 1) weapons effects on MOS related skills, 2) weapons systems environments and combat effectiveness, and 3) biomedical factors limiting soldier effectiveness. Research is conducted under field conditions and in the laboratory.</p> <p>24. (U) Animals or human subjects are subjected to conditions which produce stress of varying intensity and durations. The effects of stressors are confirmed biochemically and through observation of physiological and psychological indices. Experimental stress is then related to the ability of subjects to perform various tasks. For human subjects target acquisition and tracking, communications, endurance, and vigilance tasks are employed. Operant techniques are used with animal subjects.</p> <p>25. (U) In a recent study of helicopter pilots participating in night nap-of-the-earth training exercises, psychological, physiological and biochemical variables were evaluated. Personality factors were shown to be related to helicopter performance and to catecholamine excretion. A second field study was concerned with the measurement of blast effects on fortified positions. These data will be used to devise future investigations of the disruptive effects of weapons fire on performance efficiency. In a study of prolonged physical exercise on a bicycle ergometer certain personality characteristics were found to be related to the time to exhaustion. Physiological changes were related to subjective symptomatology subscales. Laboratory investigations of primate bloat suggest that GI tract emptying times may be useful in identifying monkeys susceptible to bloat. A study of cool-white and sunlight simulating artificial illumination revealed biological effects such as changes in weight and activity.</p>							

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1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.



# ABSTRACT

PROJECT NO.                      3M161102BS02      Basic Mechanisms of Recovery  
from Injury

WORK UNIT NO.                      065                      Military Stress and Combat  
Effectiveness

The following investigations have been conducted under this work unit:

STUDY NO. 1    Honest I - Combat Development Experimentation Command,  
Fort Ord

STUDY NO. 2    Blast effects - Electronics Command, Fort Knox

STUDY NO. 3    Assessment of physical performance

STUDY NO. 4    Environmental lighting

STUDY NO. 5    Primate acute gastric dilatation

STUDY NO. 1.    Personality characteristics, subjective fatigue, catecholamine excretion, and heart rate data were obtained from helicopter pilots engaged in night, nap-of-the-earth training exercises. Psychological test scores were related to pilot ability and to catecholamine excretion. Heart rate changes were related to urine epinephrine levels. Biochemical changes were not related to perceived anxiety as measured by psychological tests.

STUDY NO. 2.    An instrumented manikin was placed in a timber-reinforced, sand bag bunker. The physical effects of tank-fired, 105 mm projectiles which impacted at various distances from the bunker were then measured. Preliminary data suggest that occupants of reinforced bunkers would be protected against the most damaging physical effects of blast. Future studies will, therefore, be concerned with identifying weapons effects which disrupt performance in the absence of obvious injury to the subject.

STUDY NO. 3.    Subjective reports of fatigue were found to be related to changes in heart rate and  $\dot{V}O_2$  measures. A combination of the physical activities questionnaire scores, general fatigue scores, and three physiological indices of activity was used to predict total exercise duration.

STUDY NO. 4.    Hooded rats have been maintained under either normal cool fluorescent or simulated sunlight illumination. Preliminary data so far collected suggest that lighting quality affects both activity and body weight gain.



STUDY NO. 5. Physiological stressors were applied to rhesus monkeys. Behavioral and biochemical data were collected. Preliminary analyses suggest that stress induced changes in gastric emptying times may differentiate monkeys that are susceptible to acute gastric dilatation.

## BODY OF REPORT

WORK UNIT NO.	065	Military Stress and Combat Effectiveness
STUDY NO.	1	Honest I - Combat Development Experimentation Command

### PROBLEM

Nap-of-the-earth (NOE) flying by rotary wing aircraft has been proposed to be an effective means of avoiding early detection by radar. While daylight NOE flying places a greater demand on the skills of the pilot than flight at higher altitudes, these added performance requirements are further intensified at night. Training techniques to assist in preparing pilots to fly NOE at night have not yet been standardized. Information regarding factors which can affect learning to fly NOE would be helpful to persons attempting to develop such a training program.

### RESULTS AND DISCUSSION OF RESULTS

"Normal" personality factors contained in the California Psychological Inventory (CPI), subjective fatigue measured by the General States Questionnaire, urinary epinephrine and norepinephrine (bound and unbound), and heart rates of eight subject-pilots were obtained. Measurements were made during three flights which required the subjects to ride, navigate, or pilot during full moon, half moon, and no moon levels, respectively. Self Acceptance and Achievement via Independence scales of the CPI were significantly above the population mean for the pilots rated as above average in flight ability by their instructor pilots. CPI scales of Self Control and Good Impression were significantly related to urinary epinephrine, but not norepinephrine. Significant increases in epinephrine excretion were found during flights and norepinephrine between flights. No significant increases in perceived anxiety as measured by the State-Trait Anxiety Scale were noted.

### CONCLUSIONS

Psychological tests that measure "normal" personality factors may provide useful information concerning individual variation in performance while flying NOE at night. Subjective reports of stress may not necessarily be related to biochemical indices of stress. This result is related to the general adaptation syndrome.

### RECOMMENDATIONS

The study should be expanded to a larger group of pilots to affirm these findings.

## PUBLICATIONS

STAMPER, D.A., B.C. LEIBRECHT, and A.J. LLOYD. Honest I: Personality, Heart Rate, Catecholamine, and Subjective Fatigue Measures Related to Night Nap-of-the Earth Flying. Report No. 51. San Francisco, California: Letterman Army Institute of Research, April 1978

STUDY NO. 2                      Blast Effects - Electronics Command, Fort Knox

## PROBLEM

Under combat conditions, the performance of mission essential tasks will be influenced by exposure to incoming weapons fire. In the absence of physically incapacitating injuries, disruptions of performance will be primarily behavioral in nature. Such effects may be attributed to involuntary responses to the physical phenomena associated with the weapon, or may represent more complex psychological adjustments to the stresses of the combat environment. Laboratory and field investigation of the behavioral effects of exposure to weapons fire may permit the development of effective countermeasures which troops could employ in order to maintain combat effectiveness under adverse conditions. Conversely, weapons effects which produce maximum disruption of mission related performance could also be identified.

A field study planned by the US Army Electronics Command (ECOM) provided an opportunity to measure the physical characteristics of blast and projectile impacts occurring in the vicinity of a target bunker. Measurements were taken inside the bunker to assess blast effects upon a simulated human subject engaged in a target acquisition and tracking task.

## RESULTS AND DISCUSSION OF RESULTS

A timber-reinforced sand bag bunker was instrumented with sound and vibration sensors. A heavy duty tripod was equipped with a high speed camera and positioned in the bunker. The camera was focused on a series of targets located 250 m in front of the bunker and immediately adjacent to an M60 tank which would later fire on the bunker. A manikin was also installed in the bunker in the approximate position of a person using the camera equipment. The floor of the bunker, the tripod, camera base, and the head and right wrist of the manikin were equipped with accelerometers. The head of the manikin was equipped with a calibrated photodetector designed to have response characteristics similar to the human eye. An impulse noise measuring device was also installed in the bunker. Temperature sensors were attached to the dummy and at other locations within the bunker. Finally, blocks of ballistic putty and lengths of paper were placed in the bunker window to record impacts from shrapnel or other debris.

Data were collected during firing of 34 rounds of high explosive, high

explosive anti-tank, and kinetic energy 105 mm projectiles. The distances from impact point to the bunker ranged from approximately 30 m to direct hits on the bunker. In general, structural damage to the bunker was minimal. Consequently, the instruments and manikin housed inside the bunker were not subjected to direct blast effects. Preliminary data reduction suggested that peak impulse noise levels probably did not exceed 175 db. Maximum accelerations imparted to the manikin were in the 10 to 20 G range. Considerable amounts of sand and dust were blown into the bunker following some of the blasts. Dust obscuration of the targets was also evident in films taken from within the bunker. Although occasional impacts of shrapnel and other debris were registered in the window area, there was no evidence that these materials reached the inside of the bunker. None of the rounds produced changes in the temperature sensors located inside or outside the bunker. Photo-detector data have not been analyzed.

#### CONCLUSIONS

Further data analysis will be required to define precisely the ranges of physical phenomena encountered during this study of blast effects. The resulting data will permit formulation of new laboratory and field studies to describe the effects of weapons fire on the performance of mission essential tasks. Preliminary data suggest that the timber reinforced sand bag bunkers afforded considerable protection against 105 mm tank fired projectiles. Although potentially hazardous sound levels were encountered, the results also suggest that physical injuries to occupants of reinforced bunkers would be minimal under the conditions of this experiment. However, appreciable disruptions of task performance might accompany the physiological and behavioral responses of individuals exposed to otherwise non-lethal weapons effects.

#### RECOMMENDATIONS

Further research is needed to measure deterioration of combat effectiveness attributable to weapons effects other than physical incapacitation.

#### PUBLICATIONS

None

STUDY NO. 3                      Assessment of physical performance

#### PROBLEM

To complete a prolonged physical activity task, a soldier must necessarily rely on feedback mechanisms which enable him to adjust the amount of work he is performing relative to the period of time over which it must be performed. The feedback process includes physiological



changes, subjective symptomatology, and personality factors. Physiological changes associated with prolonged physical work include both general and specific effects. The general effects can be observed in a wide variety of work situations while specific effects occur in response to the unique demands of each task. The subjective symptomatology associated with each task seems to reflect the physiological changes that accompany physical work. Subjective symptoms, therefore, are probably influenced by both general and specific factors. Various personality models have been proposed to account for performance differences in a variety of situations. Several models, based on an inhibition-satiation phenomenon, have shown some success in predicting individual performance differences. The inhibition-satiation phenomenon is a process that serves to modify the perception of somatosensory information. Modification of the perception of stimuli must be evaluated.

#### RESULTS AND DISCUSSION OF RESULTS

Subjective estimates of fatigue, physiological, and personality data were collected from fourteen male college students during two rides on a bicycle ergometer performed at 65% and 85% of  $\dot{V}O_2$  Max level. Subjective estimates of leg fatigue and general fatigue as measured by the Physical Activity Questionnaire (PAQ) increased significantly from three minutes to the end-of-ride. Significant relationships were also found between these subscales and electromyographic recordings and cardiopulmonary variables. The same three minute to end-of-ride comparison made for the Cardiopulmonary subscales indicated that the subjects were not uniformly affected by the work. For this subscale, the scores for the short duration riders (<16 minutes) and long duration riders (>32 minutes) increased significantly, but scores for intermediate duration riders (16 to 32 minutes) did not. Heart rate data showed the same pattern of change across the groups. The Motivation subscale showed essentially no change throughout the ride. This finding would appear to be related to the pre-ride instructions to the subjects which were intentionally not highly motivating.

Results of a series of multiple regression analyses of the physiological data obtained from the 12-minute data collection period indicated that (1) 12-minute score based on the percent of 3-minute score was a better predictor of ride duration ( $R = 0.94$ ) than the absolute value at 12 minutes ( $R = 0.55$ ); (2) General Fatigue and Cardiopulmonary subscales in combination was a good predictor ( $R = 0.80$ ) of ride duration while Leg Fatigue and Motivation levels provided little additional predictive power; and (3) a combined analysis (PAQ subscale, and General Fatigue with three physiological variables) could be used to predict total ride duration at an extremely high confidence level ( $R = 0.99$ ,  $P < 0.001$ ). Apparently, some additional variability related to feelings of general fatigue experienced while performing this work task has yet to be specified.

Only one subscale from the three personality tests, i.e., the Disinhibition subscale of the Sensation Seeking Scale (SSS-DIS), was related to total ride time. However, contrary to a proposed model based on the inhibition-satiation phenomenon, the relationship was negative. Specifically, individuals that performed best on this physical endurance task were those who tended to show the lowest scores (the more inhibited persons). This suggests inadequacies in the proposed model. Perhaps specific psychosocial, work intensity, and stimulus intensity factors must also be considered.

#### CONCLUSIONS

With the data available, it does appear that subjective reports of fatigue are related to certain physiological changes such as change in heart rate, ventilation, etc. Also, subjective reports of general fatigue in combination with certain physiological variables can be highly predictive of total ride duration. Lastly, personality factors that are based on the inhibition-satiation phenomenon are related to performance differences among individuals; however, the relationship is negative.

#### RECOMMENDATIONS

The findings of this study should be reevaluated with a larger group of subjects to test the generality of the findings.

#### PUBLICATIONS

1. WEISER, P.C. and D.A. STAMPER. Psychophysiological interactions leading to increased effort, by fatigue, and respiratory distress during prolonged strenuous bicycle riding. In: Physical Work and Effort, Volume 38, edited by G. Borg. New York: Pergamon Press, 1976.
2. STAMPER, D.A. Physiological, Psychological, and Symptomatic Factors Affecting Prolonged Physical Performance. (Submitted for review and clearance as LAIR laboratory report).

STUDY NO. 4            Environmental lighting

#### PROBLEM

Various stressors encountered in warfare may alter susceptibility to and recovery from disease. Such illness and disease may significantly reduce the performance of MOS related skills. Reports suggest that the quality of artificial lighting may influence the susceptibility of the individual to infectious diseases. The purpose of the present investigation is to explore some readily identifiable behavioral or biological effects of light in order to establish that such effects do exist or do not exist.

## RESULTS AND DISCUSSION OF RESULTS

Twelve hooded rats were divided into two groups. One group was maintained under normal cool-white fluorescent lighting (cool white), the other group was maintained under Vita Lite fluorescents which more closely approximate the spectrum of sunlight. Half of each group was further subdivided into a normal and a stress group. Stress was provided by random burst of white noise. A replication is planned.

Preliminary results suggest that activity levels and growth (weight gain) are influenced by the spectral content of the ambient illumination.

## CONCLUSIONS

None

## RECOMMENDATIONS

Completion of this study is recommended. In addition, it is suggested the relationship between light and susceptibility to disease be directly explored.

## PUBLICATIONS

None

STUDY NO. 5                      Primate acute gastric dilatation

## PROBLEM

Spontaneous acute gastric dilatation (bloat) in the primate is a serious problem in the research laboratory because it usually leads to death. Primate bloat, therefore, disrupts experiments, requires intensive veterinary support, and consumes a valuable animal resource. What little is known about the cause of the syndrome suggests that a combination of stressful conditions and/or feeding procedures may play a major etiologic role. Individual organismic factors have also been implicated. A major purpose of the study is to determine the behavioral, psychophysiological and stress factors which may play a role in primate bloat. The results of this approach will contribute to the conservation of valuable primate resources within the military laboratory.

Progress in studying the bloat syndrome has been hampered by the lack of a reliable experimental paradigm. Accordingly, the second major objective is to establish a non-surgical technique for reproducibly producing bloat in rhesus monkeys.



## RESULTS AND DISCUSSION OF RESULTS

Four experimental manipulations were used to study stress responses in rhesus monkeys: altered feeding schedule, altered lighting schedule, handling by an unfamiliar caretaker, and altered social interactions through rearranged caging. Collection of behavioral and biochemical data was completed, but no incidents of acute gastric dilatation occurred during the period of study. According to preliminary analysis, measures of behavioral reactivity and changes in urinary 17 OH-ketosteroids failed to differentiate between monkeys that have previously bloated and those who have not. Although not statistically significant, the data suggest that gastric emptying of the two groups may change differentially during stress. An analysis of urinary free cortisol excretion is in progress.

## CONCLUSIONS

Although the stress manipulations did not induce bloat, this is not conclusive regarding the involvement of stress in the etiology of bloat. With the exception of gastric emptying times, the behavioral and biochemical measures which have been analyzed do not appear to correlate with previous history of bloat.

## RECOMMENDATIONS

Stress induced changes in gastric emptying times should be studied further to determine whether or not they correlate with susceptibility to bloat and could be used as predictors.

## PUBLICATIONS

None

## OTHER PUBLICATIONS PREPARED BY GROUP

LEIBRECHT, B.C., A.J. LLOYD, and P.A. O'MARA. Field Study of Stress: Psychophysiological Measures During Project SUPLEX. LAIR report (submitted for clearance).



AD-A066 619

LETTERMAN ARMY INST OF RESEARCH SAN FRANCISCO CALIF  
U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT TECHNICAL REPORT. (U)  
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUM. <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS		9. LEVEL OF SUM
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10. NO./CODES <sup>a</sup>		PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		61102A	3M161102BS02		00		066	
b. <del>XXXXXXXX</del>								
c. <del>XXXXXXXX</del>		CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>								
(U) Physical, Chemical Characteristics of Human Stratum Corneum								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>								
003500 - Clinical Medicine								
13. START DATE			14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
67 07			CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS		B. FUNDS (in thousands)
a. DATES/EFFECTIVE:				PRECEDING				
b. NUMBER <sup>a</sup>				FISCAL		77		2.6
c. TYPE: Not Applicable				YEAR		CURRENT		64
d. KIND OF AWARD:						78		2.6
e. CUM. AMT.								55
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
NAME <sup>a</sup> : Letterman Army Institute of Research				NAME <sup>a</sup> : Letterman Army Institute of Research				
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:				
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS				
				NAME:				
				NAME:				
				POC: DA				
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>								
(U) Stratum Corneum; (U) Absorption; (U) Permeability; (U) Water; (U) Water Vapor; (U) Chemicals; (U) Persistence; (U) Skin; (U) Human Volunteers								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) The objective is to define the physical-chemical characteristics of the stratum corneum and its interaction with water, chemicals, ultraviolet radiation, and environment. These characteristics are fundamental to the etiology of several epidemic dermatological disorders caused by exposure of the soldier's skin to the environment and are also applicable to the behavior of topical preparations in human skin.								
24. (U) A model is under development for the measurement and kinetic analysis of the hydration state of stratum corneum under varying conditions of temperature, relative humidity, and wind speed simultaneously to be used for evaluating skin protective agents. In vitro measurements of skin permeability and evaporation rates of insect repellents, skin waterproofing agents, antifungal compounds, and sunscreens continue to provide guidelines for formulating compounds and vehicles to extend the duration on the skin and thereby increasing protection.								
25. (U) 76 10 - 77 09. A thermogravimetric apparatus is being modified to provide more reliable and accurate determination of the interrelationship of temperature, relative humidity, and wind velocity as they affect the hydration state of stratum corneum. Evaporation-penetration experiments were accomplished for 2 mosquito repellents and a sunscreen. Evaporation of deet from skin has been compared in vivo and in vitro.								

# ABSTRACT

PROJECT NO. 3M161102BS02 Basic Mechanisms of Recovery from Injury

WORK UNIT NO. 066 Physical, Chemical Characteristics of Human Stratum Corneum

The following investigations have been conducted under this work unit:

STUDY NO. 1 Hydration kinetics of the human stratum corneum

STUDY NO. 2 Percutaneous absorption and evaporation of topical agents applied to the skin

STUDY NO. 3 Evaporation of insect repellents in vivo and in vitro

STUDY NO. 1 An apparatus consisting of a quartz beam thermogravimetric device coupled to a thermoevolution analyzer recorder is being developed to obtain measurements of stratum corneum hydration as influenced by varying temperature relative, humidity, and air speed.

STUDY NO. 2 The percutaneous absorption and evaporation of 2 repellents, a sunscreen and an essential fatty acid were determined.

STUDY NO. 3 The minimum effective concentration of the repellent deet was determined both in vitro and in vivo with a variety of techniques. These various determinations were in agreement with each other and the value of  $3.5 \mu\text{g}/\text{cm}^2/\text{h}$  was found as the minimum effective evaporation rate for deet.

## BODY OF REPORT

WORK UNIT NO. 066

Physical, Chemical Characteristics of  
Human Stratum Corneum

STUDY NO. 1

Hydration kinetics of the human  
stratum corneum

### PROBLEM

During the Vietnam campaign, U.S. Army personnel sustained 260,000 man-days lost due to skin diseases from 1967 through 1971. Prolonged exposure to water and increased temperature of the tropical environment played a major role in this manpower loss. Excessive hydration of the stratum corneum or dehydration breaks down the normal barrier foundation of the stratum corneum. Development of an in vitro model to study hydration kinetics is essential to the development of hydrophobic skin protective agents.

### RESULTS AND DISCUSSION OF RESULTS

In past work at LAIR, techniques have been developed to isolate and store stratum corneum successfully. An apparatus consisting of quartz beam thermogravimetric device coupled to a thermoevolution analyzer recorder is being developed to obtain the measurements of hydration state of the stratum corneum. The entire apparatus is housed in a temperature controlled environment. Auxiliary equipment to control humidity and wind speed have also been developed. This experimental design will permit the measurement of hydration state of stratum corneum as it is simultaneously influenced by temperature, relative humidity, and wind speed.

### CONCLUSIONS

This apparatus will provide measures of the hydration state of the stratum corneum as it is influenced by temperature, relative humidity, and air flow.

### RECOMMENDATIONS

We should continue to make measurements of stratum corneum hydration to insure the validity of the model.

### PUBLICATIONS

None

STUDY No. 2

Precutaneous absorption and evaporation  
of topical agents applied to the skin



## PROBLEM

Stratum corneum, the outermost layer of the skin, has been recognized as the protective barrier against topically applied drugs, cosmetics, detergents, solvents, and other chemicals which sometimes contact the skin at high concentrations. However, during the last decade, there have been reports that a portion of the topically applied dose of a variety of compounds may be absorbed through the stratum corneum. To evaluate these reports and to study the protective role of the stratum corneum, we designed a method to measure both the evaporative and penetrative losses of substances from the skin as well as the total amount of the compound penetrating the skin if it remains on the surface over an extended period of time. Our findings indicate that a proportion of certain compounds penetrate and that the portion which penetrates is similar for compounds applied at high concentrations and low concentrations.

## RESULTS AND DISCUSSION OF RESULTS

In model systems developed to study loss of chemicals from the skin, the most common approach has been permeability chambers (cells) with either whole skin, separated epidermis, or stratum corneum. Although systems, with a liquid phase on both sides of the membrane, yield steady state penetration data with accurate physical constants, the membrane is over-hydrated; therefore, the liquid/liquid system is not intended to duplicate in vivo conditions. Permeability cells which have an aqueous phase only on the dermal side of the membrane give a closer approximation to in vivo penetration; however, loss of substances from the skin frequently occurs by both evaporation and penetration.

The cell used in the current study is designed to permit detection of both evaporation and penetration through a piece of excised skin. Ringer's lactate solution flows under the dermal side, and dry air passes over the stratum corneum surface where the test compound is applied. In our early work with insect repellents in the evaporation-penetration cell, we found that excessive hydration of the corneum resulted in less penetration and more evaporation than we observed later when we used intact skin in place of stratum corneum. Apparently, the dermal layer of whole skin protects the stratum corneum from excessive hydration while it acts as a lipophilic sink for the repellent compounds. Although the preceding observation appears to contradict the premise that greater hydration means greater skin permeability, the decreased permeability in this instance simply reflects the tendency of an oily repellent to remain on the surface rather than to penetrate into the water phase of an excessively hydrated membrane. Normally, skin maintains a lipid-water balance into which an oily compound would penetrate.

In our studies with the evaporation-penetration cell, we found that

mosquito repellents persisted on the excised skin surface for approximately the same period of time as repellents protected men against mosquitoes in vivo. Over a 24 h period, approximately 50% of the most commonly used mosquito repellent N,N-diethyl-m-toluamide (deet) evaporated from the skin surface while 34% penetrated. With a second experimental repellent compound, carbamide, only 6% was lost by evaporation while 70% penetrated. The penetration of repellents in vitro can be used to estimate the amount of compound penetrating in vivo. If a moderate dose of the repellent deet ( $0.3 \text{ mg/cm}^2$ ) is applied to the arms and face ( $3000 \text{ cm}^2$ ), the absorbed dose would be about 270 mg per application in 24 h in still air. The absorbed dose varies with the size of the body area, the site, and the length of time the repellent persists on the skin.

The same absorption principle has been tried in treatment of essential fatty acid (EFA) deficiency in patients with long-term total parenteral nutrition. Safflower oil was applied to the skin surface to provide sufficient linoleic acid ( $\text{C}_{18}$ ) to prevent EFA deficiency. In our studies, a similar compound, hexadecanoic acid ( $\text{C}_{16}$ ), had about 30% penetration in the evaporation-penetration cell. If a dose of  $\text{C}_{16}$  ( $1 \text{ mg/cm}^2$ ) was applied over the arms and back ( $5000 \text{ cm}^2$ ), as much as 1500 mg would be absorbed over a 24 h period.

In our in vitro system, 23% of the sunscreen p-amino-benzoic acid (PABA) penetrated the skin. Although 23% is not a small proportion, PABA formulations are designed to maintain the active compound on or in the stratum corneum. Moreover, the necessary concentration of PABA is low (5%), which favors less total PABA penetration in ordinary use.

#### CONCLUSIONS

In vitro data indicate that evaporation from the skin surface is a major mode of loss for certain insect repellents. In addition, substantial amounts of insect repellents and other compounds penetrate into the skin.

#### RECOMMENDATIONS

Studies of percutaneous absorption and evaporation (where appropriate) of agents of interest to the military should be continued.

#### PUBLICATIONS

None

STUDY NO. 3

Evaporation of insect repellents in vivo  
and in vitro

## PROBLEM

Although percutaneous absorption has been considered in many studies, certain substances like mosquito repellents are lost in large part by evaporation from the skin surface. Since vapor-phase repellents act by disrupting the mosquito in her approach to the skin, evaporative loss is necessary to maintain at least the minimal concentration of repellent above the skin which will repel mosquitoes. This concentration is called the minimum effective concentration (MEC) and can be used to estimate the intrinsic repellency of the repellent chemical.

## RESULTS AND DISCUSSION OF RESULTS

The parameter which has been measured is the MEC above the skin or the minimum effective evaporation rate of repellent from the skin which is necessary to repel mosquitoes. The minimum effective applied dose of  $25 \mu\text{g}/\text{cm}^2$  was determined previously by application of the repellent deet in successively lower doses on 16 individuals until the repellent no longer afforded protection when the treated forearm was exposed to mosquitoes. Comparison of the evaporation<sup>2</sup> rates determined by two different in vivo methods yields  $3.5 \mu\text{g}/\text{cm}^2/\text{h}$  as a good approximation to the minimum evaporation rate. This compares favorably to a minimum effective evaporation rate of  $5 \mu\text{g}/\text{cm}^2/\text{hr}$  found in vitro. The MEC can be calculated from the area of evaporation and flow rate, giving a result of  $1.16 \text{ ng/ml}$  or  $6.1 \times 10^{-10} \text{ moles/liter}$ . These levels of steady state evaporation are comparable to the evolution of certain normal body effluents determined in previous studies; hence, some natural human factors are competitive with repellent vapors at repellent doses approaching the minimum effective levels. This observation is in agreement with previous studies showing that differences between individuals have significant influence on the duration of repellent efficacy.

Repellent loss from the skin at the low dose considered here appears to follow simple first-order kinetics, i.e., the evaporation rate decreases logarithmically with respect to time. However, at higher doses in vitro and in vivo a steady state evaporation rate has been observed. We also observed considerable differences in evaporation rates at different sites on the same and different individuals.

The biological variation in adjacent skin sites has been recognized in previous reports. Despite this variation observed both in vivo and in vitro, the relative portion of repellent evaporating in each case is similar.

## CONCLUSIONS

There are differences in the measured evaporation rate of a repellent applied at the minimum effective dose. Moreover, on different



individuals the resulting MEC of repellent above the skin is comparable to the concentration of some normal body effluents. Consequently, individual differences affecting either relative evaporation and penetration and the normal body effluents may affect the efficacy or persistence of a topical agent like a repellent.

#### RECOMMENDATIONS

This study supports the validity of our in vitro techniques and the in vitro work should continue as a reliable means of assessing minimum effective evaporation rates of repellents. Information of this type is vital to formulation work so that the necessary amount of repellent evaporation is allowed, while excessive evaporation is reduced.

#### PUBLICATIONS

1. AKERS, W.A., and T.S. SPENCER. Drug loss from the skin by evaporation and penetration. Scientific exhibit, American Academy of Dermatology, Chicago, December, 1976. Received the Gold Award for original scientific work.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY 77 05 06	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>a</sup> NA	8A. DISSEM INSTR <sup>a</sup> NL	8B. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO. / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102A	3W161102BS02	00	067			
<del>XXXXXXXXXX</del>							
<del>XXXXXXXXXX</del> CAPDS 114 f							
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Biochemical Mechanisms of Pathogenesis in Fungal Skin Infections							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002300 - Biochemistry; 010100 - Microbiology; 003500 - Clinical Medicine							
13. START DATE 73 09	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House		
17. CONTRACT GRANT		18. RESOURCES ESTIMATE					
A. DATES/EFFECTIVE:		EXPIRATION:		PRECEDING		B. PROFESSIONAL MAN YRS	
B. NUMBER <sup>a</sup>		C. TYPE: Not Applicable		FISCAL YEAR		D. FUNDS (In thousands)	
E. KIND OF AWARD:		F. CUM. AMT.		CURRENT			
				77		3	
				78		95	
						98	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				Department of Dermatology Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5455			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Jaeger, June, DAC			
				NAME: Foye, Jack, SFC			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup> (U) Skin; (U) Pathogenicity; (U) Enzymes; (U) Vaccine; (U) Fungal Inhibitors							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) (U) Under unfavorable conditions, epidemics of fungal infections can incapacitate 38 percent of combat soldiers within 16 days of first exposure with the average period of incapacitation 7.3 days. The biochemical pathways fungi use to attack skin will be investigated. The effects of varying environmental factors on invasion by fungi in man's skin will be determined - temperature, water, humidity, occlusion. Novel preventive and therapeutic measures will be sought.							
24. (U) The approach will be to (1) evaluate effects of environmental factors and nutritional factors on germination and growth of dermatophytes in vitro, (2) develop a method to prepare suspensions of individual arthrospore, and (3) evaluate the role of exocellular enzymes in experimental fungal infections.							
25. (U) 76 10 - 77 09. Purified single cell arthrospores have been prepared from <u>T. mentagrophytes</u> and <u>T. rubrum</u> . Antibodies have been prepared against exocellular enzymes for use in fluorescent antibody techniques. Large quantities of trichophytin antigen have been secured and are presently under evaluation. Electrophoretic and iso-electrofocusing methods were developed to separate trichophytin. Alcian-blue and periodic-acid-Schiff stains were utilized to visualize the antigen and to monitor various purification steps.							

# ABSTRACT

PROJECT NO.	3M161102BS02	Other Tropical Medicine
WORK UNIT NO.	067	Biochemical Mechanisms of Pathogenesis in Fungal Skin Infections

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Preparation of purified skin test antigens of the dermatophytes
- STUDY NO. 2 Morphological and chemostructural characterization of the events of ontogenetic development of Trichophyton mentagrophytes

Both studies are designed to determine mechanisms of pathogenesis of dermatophytes.

STUDY NO. 1 The first study concentrates on development of antigens useful in studies of immune response to dermatophyte infection. Production of a quantity of antigen and production of antisera against trichophyton were two major goals of the study. Both were accomplished.

STUDY NO. 2 The second study seeks to determine the relationship of morphologically distinct fungal forms to the events of fungal infection. A model system, with the use of chorioallantoic membrane of chicken eggs as a representation of epidermal tissue, has been developed. Successful production in vitro of individual arthrospores have allowed investigations of the role of these morphologic forms to begin.

## BODY OF REPORT

WORK UNIT NO. 067

Biochemical Mechanisms of Pathogenesis in Fungal Skin Infections

STUDY NO. 1

Preparation of purified skin test antigens of the dermatophytes

### PROBLEM

Dermatophyte infections are prominent producers of medically debilitating skin lesions in soldiers. The predominant etiologic agent for such infections in U.S. Army personnel in the Republic of Vietnam has been identified as Trichophyton mentagrophytes var. granulare. Although there is a voluminous amount of literature written about these fungal infections, a paucity of information remains regarding the specific mechanisms of pathogenesis. Research from this study has been centered upon preparation and characterization of trichophytin antigens. Preparation of adequate quantities of antigen will allow further investigation into the mechanism of pathogenesis due to the host immune response.

### RESULTS AND DISCUSSION OF RESULTS

Conditions for growth of fungi and processing of antigen were optimized. Growth of T. mentagrophytes var. asteroides in a complex medium in a well-aerated fermenter consistently produces high yields of trichophytin antigen. A series of column chromatography procedures were developed which are capable of resolving trichophytin antigen into a single component. Our most highly purified trichophytin as well as T. mentagrophytes spores and T. mentagrophytes hyphal mat were used as antigen to immunize rabbits. Good antibody response was detected in all 3 groups of rabbits. As expected, sera from rabbits immunized with trichophytin formed precipitin lines with either spores or hyphal mat. Conversely, sera from rabbits immunized with either spores or hyphal mat reacted with trichophytin. These immune sera are to be used for a variety of investigations. Investigations will include fluorescent antibody detection of trichophytin in active infection, detections of host antibodies toward T. mentagrophytes, and affinity chromatography purification of trichophytin.

One of our major objectives has been to procure a single large lot of trichophytin antigen. The lot was produced by an outside contractor to our specification. Activity assayed in guinea pigs is 25 µg/skin test produced lesion  $14 \pm 0.9$  mm at 24 h; 10 µg/skin test produced lesion  $10 \pm 0.6$  mm at 24 h. These reactions are equivalent to those produced by small lots of trichophytin produced in our laboratory. Total yield was approximately 4.0 g or approximately 400,000 ten µg doses. The antigen produces a precipitin line of



equivalence when assayed with homologous trichophytin and rabbit anti-Trichophyton sera.

### CONCLUSIONS

Two major goals of this study were realized. A single large lot of trichophytin was procured. Antisera against both trichophytin and whole live fungal organisms were produced. Meeting these goals has provided tools for expansion of the study into investigation of the role of specific antigens in fungal infection.

### RECOMMENDATIONS

Although trichophytin has been produced in quantity and appears to be the major antigenic component of cultured T. mentagrophytes, evidence is still lacking to indicate that trichophytin plays a major role in eliciting immunity to fungal infection. Other antigens isolated either from infections, prepared from fungi grown on stratum corneum, or considered to be invariant among morphologic forms will need to be isolated and tested for immune capacity. Specific antisera against trichophytin will be useful in delineating the location of trichophytin in infection. Fluorescent labeling techniques will provide some basis to determine depth of penetration of antigen and site of immune reactions.

### PUBLICATIONS

1. LANCASTER, M., J.C. FOYE, and J.S. JAEGER. Visualization of the trichophytin antigen from Trichophyton mentagrophytes utilizing the technique of isotachopheresis. (Abstract) In: Annual Meeting of American Society for Microbiology (New Orleans, LA., 8-13 May 1977), p 378

STUDY NO. 2

Morphological and chemostructural characterization of the events of ontogenetic development of Trichophyton mentagrophytes

### PROBLEM

Determining how saprophytic septate branching mycelium changes to the pathogenic hypersegmented mycelium or arthrospore form will lead to the identification of the conditions causing this alteration and its accompanying enzyme systems. As they become known, this will permit altering the physical conditions of the host or blocking some of the enzyme systems by chemotherapy which may be prophylactic or therapeutic measures against cutaneous fungal infections which molest our combat troops.



## RESULTS AND DISCUSSION OF RESULTS

Preliminary results from inoculating the chorioallantoic membrane (CAM) of a fertile chicken egg indicate that this is a good media for studying the conversion of microconidia to hypersegmented mycelia. The conversion occurs in less than 5 days. It was observed by scanning electron microscopy that the surface of the mycelia excretes a material that forms fine strands which intermesh and attach to other hyphae and host cells. An inflammatory reaction is elicited by the host tissue. These fine strands play a role in the pathogenic process. They appear to trap some blood cells in the CAM by forming a meshwork. The excretion of these strands have been duplicated by growing small fungal colonies on the sides of glass tubes after preincubating spores in a mannitol salts urea media for 1 week. Growing Trichophyton mentagrophytes var. granulare (ATCC 18748) on a solid media of this composition causes the fungus to convert to the hyper-segmented form with some stranding but greater amounts of stranding are observed on the glass surface above the medium. It is not produced by, at least, 2 strains of T. rubrum and one strain of T. interdigitale. Light microscopy tissue sections revealed the CAM was invaded throughout all 3 layers with the ATCC 18748 strain.

## CONCLUSIONS

During the course of this study it has become clear that there are events occurring in a host tissue during a dermatophyte invasion that were not observed before. (We can now produce more of this stranding or fibrous material and can determine its role in an infection.)

## RECOMMENDATIONS

A time study of the process of invasion of CAM should be undertaken to observe more closely how long it takes the microconidia to germinate and how they penetrate. Only by observing these details by electron microscopy (and perhaps there are some yet undiscovered ones), can we duplicate this growth in vitro and isolate the enzyme systems involved. Chemical identification of the fibrous material excreted through the walls should be undertaken to determine its role in pathogenesis.

## PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 77 07 19	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>a</sup> NA	8a. DISSEM INSTR <sup>a</sup> NL	8b. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3M161102BS02	00	070			
b. CONTRIBUTING							
XXXXXXXXXX CARDS 114 f							
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Influence of Nutrients, Hormones and Related Substances on the Recovery from Injury.							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002300 Biochemistry; 012900 Physiology; 002600 Biology							
13. START DATE 76 10		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: Not Applicable				PRECEDING 77		2.0	
b. NUMBER: Not Applicable				FISCAL YEAR		137	
c. TYPE: Not Applicable				CURRENT 78		2.0	
d. KIND OF AWARD: Not Applicable						182	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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ADDRESS: <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS: <sup>a</sup> Department of Medicine Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J. E., COL, MC				NAME: <sup>a</sup> Bikle, D. D., MAJ, MC			
TELEPHONE: 415-561-3600				TELEPHONE: 415-561-4147			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Herman, R. H., COL, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Fracture of bone; (U) Stress fracture; (U) March fracture; (U) Non-union of bone; (U) Mal-union of bone; (U) Vitamin D metabolism; (U) Small intestine.							
23. (U) Military personnel in training and combat incur a high incidence of march (stress) and traumatic fractures which cause much morbidity and disability. March fractures often heal poorly; 30 percent of patients never return to duty. A large percentage of traumatic fractures heal more slowly than normal or not at all. We will determine if the metabolism of vitamin D is abnormal in these patients and responsible for the poor healing of bone.							
24. (U) The metabolism of vitamin D will be studied in patients of military age with march fractures and in animals. Small intestinal mucosa obtained via peroral biopsy in the patients will be incubated in vitro and the uptake of calcium and phosphate, the activity of alkaline phosphatase and ribonucleic acid (RNA) polymerase, and the concentration of calcium binding protein will be determined. These parameters will be measured while the patients are on low and high calcium diets. The status of bone will be determined by appropriate x-rays, osteodensitometry, bone biopsy, and ultra-sound measurements. The levels of 25-hydroxy vitamin D <sub>3</sub> , 1,25 dihydroxyvitamin D <sub>3</sub> and parathormone will be determined. Parallel studies will be done in rachitic chicks.							
25. (U) 76 10 - 77 09 Using the rachitic chick as a model, we have evaluated the effect of 1,25 dihydroxyvitamin D <sub>3</sub> and its synthetic analog, 1α-hydroxyvitamin D <sub>3</sub> on the following intestinal and bone parameters: calcium mobilization and transport, phosphate mobilization and transport, calcium binding protein, alkaline phosphatase, RNA, and protein synthesis. We have demonstrated that calcium transport and alkaline phosphatase, in contrast to calcium mobilization and calcium binding protein, are stimulated by 1,25 dihydroxyvitamin D <sub>3</sub> and 1α-hydroxyvitamin D <sub>3</sub> without de novo RNA induction. We have found that the calcium content of the diet not only alters calcium uptake in tissue obtained by gut biopsy but also alters the ability of 1,25 dihydroxyvitamin D <sub>3</sub> to stimulate calcium binding protein and calcium uptake.							

#### ABSTRACT

PROJECT NO.	3M161102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	070	Influence of Nutrients, Hormones and Related Substances on the Recovery from Injury

The following investigations have been conducted under this work unit:

STUDY NO. 1    The effect of 1,25 dihydroxyvitamin  $D_3$  and  $1\alpha$ -hydroxyvitamin  $D_3$  on calcium activated ATPase, calcium binding protein, and calcium and phosphate uptake in the chick intestine.

The relationship between 1,25 dihydroxyvitamin  $D_3$  ( $1,25(OH)_2D_3$ ) mediated calcium transport, calcium binding protein (CaBP), and alkaline phosphatase (alk Pase) has been explored. Our data indicate that calcium transport is less influenced by CaBP and alk Pase than by dietary calcium and phosphate for the following reasons: (1) Calcium transport is stimulated earlier following  $1,25(OH)_2D_3$  administration than CaBP or alk Pase; (2) Inhibition of CaBP or alk Pase induction of protein synthesis inhibitors does not prevent stimulation of calcium transport; and (3) Increasing dietary calcium enhances CaBP induction blocks alk Pase activation and has a biphasic effect on calcium transport.

The ability of  $1,25(OH)_2D_3$  to stimulate calcium transport via an action on mitochondria and microsomes was examined, but no direct influence could be demonstrated.  $1,25(OH)_2D_3$  appears to stimulate calcium transport by an action on the plasma membrane, an effect regulated by dietary calcium and phosphate, but it is not dependent on protein synthesis.



## BODY OF REPORT

WORK UNIT NO. 070

Influence of Nutrients, Hormones  
and Related Substances on the Recovery  
from Injury

STUDY NO. 1

The effect of 1,25 dihydroxyvitamin  $D_3$   
and  $1\alpha$ -hydroxyvitamin  $D_3$  on calcium  
activated ATPase calcium binding  
protein, and calcium and phosphate  
uptake in the chick intestine.

### PROBLEM

Two types of fractures occur with high frequency in the military population: (1) the fracture due to impact (e.g. missile wounds, sudden blow to a bone), and (2) the stress fracture (also called march or fatigue fracture). Both types of fractures cause considerable morbidity and prolonged, if not permanent, disability in the affected soldier. Recent advances in the understanding of the mechanism of action of vitamin D make it possible to investigate the potential role of vitamin D in the etiology and treatment of these bone problems. These animal studies represent the investigation of in vitro techniques which, if successful, will be applied to the study of patients with these and related types of bone disease. We have investigated the mechanism whereby 1,25 dihydroxyvitamin  $D_3$  ( $1,25(OH)_2D_3$ ), the biologically active metabolite of vitamin  $D_3$ , regulates calcium transport across the gut. We have determined that this process does not require de novo protein synthesis, in particular synthesis of CaBP and alk Pase. We have found, however, that dietary concentrations of calcium and phosphate alter the influence of  $1,25(OH)_2D_3$  on the gut which suggests that such dietary factors cannot be ignored in one's evaluation of soldiers with fractures.

### RESULTS AND DISCUSSION OF RESULTS

Stimulation of calcium transport by  $1,25(OH)_2D_3$  is observed within 2 hours after in vivo administration of the hormone. Induction of CaBP and activation of alk Pase require 4 to 6 hours. Both the induction of CaBP and the activation of alk Pase can be blocked by cycloheximide; the stimulation of calcium transport is not affected. The paradoxical stimulation of alk Pase by actinomycin D, which is additive to that by  $1,25(OH)_2D_3$  or  $1\alpha OH D_3$ , is also inhibited by cycloheximide.

Calcium accumulation by duodenal mucosa in vivo following  $1,25(OH)_2D_3$  administration is biphasic with maximal accumulation occurring at the onset of CaBP synthesis and diminishing as CaBP concentrations increase. The bulk of calcium inside the cell is found in mitochondria. If CaBP synthesis is blocked by cycloheximide, the gut mucosa continues to accumulate calcium following  $1,25(OH)_2D_3$  with a massive increase in mitochondrial granules.



Under conditions of fixed  $1,25(\text{OH})_2\text{D}_3$  dosage a marked influence of dietary calcium and phosphate on CaBP, alk Pase, and calcium transport was observed. Increasing calcium content of the diet led to increased CaBP production but decreased alk Pase stimulation. The effect of phosphate on CaBP production is less impressive, but it has a similar influence to calcium on alk Pase. Acute changes in diet show a biphasic effect on calcium transport with 1.2% calcium inhibiting compared to 0.12% or 2-32% calcium and 0.65% phosphate stimulating compared to 0.25% or 1.2% phosphate. After 4 days on the diet, the influence on transport was less pronounced. Intestinal mitochondria from chicks given  $1,25(\text{OH})_2\text{D}_3$  were compared to those from rachitic chicks in their ability to take up calcium. No difference was seen either in extent of accumulation or in concentration of calcium required to observe accumulation. No difference was observed in the ability of the intestinal cytosol from the  $1,25(\text{OH})_2\text{D}_3$  treated vs. rachitic chicks to stimulate calcium uptake by the mitochondria.

#### CONCLUSIONS

1. The stimulation of calcium transport across the gut by  $1,25(\text{OH})_2\text{D}_3$  does not require the induction of CaBP or alk Pase.
2. Dietary calcium and phosphate modulate the effects of  $1,25(\text{OH})_2\text{D}_3$  on the gut.
3. The effect of  $1,25(\text{OH})_2\text{D}_3$  on calcium transport is probably at the level of the plasma membrane rather than the mitochondrion.

#### RECOMMENDATIONS

The means by which calcium and phosphate regulate  $1,25(\text{OH})_2\text{D}_3$  action on the gut should be explored further and the effects of  $1,25(\text{OH})_2\text{D}_3$  on the plasma membrane should be studied.

#### PUBLICATIONS

1. BIKLE, D. D., D. T. ZOLOCK, R. L. MORRISSEY, and R. H. HERMAN. The dissociation of  $1,25(\text{OH})_2\text{D}_3$ -induced CaBP production and alkaline phosphatase activity from calcium transport by actinomycin D and cycloheximide. In: Vitamin D: Biochemical, Chemical, and Clinical Aspects Related to Calcium Metabolism, edited by A.W. Norman, J.W. Coburn, H.F. DeLuca, D. Fraser, H.G. Grigoleit, and K. Schaefer. Elmsford, NY: Walter deGruyter, Inc, 1977, pp 223-225.
2. CHARLES, M. A., J. MARTIAL, D. ZOLOCK, R. MORRISSEY, D. BIKLE, and J. BAXTER.  $1,25$ -dihydroxycholecalciferol stimulation of calcium binding protein specific m-RNA. *ibid.* pp 227-229.
3. ZOLOCK, D. T., R. L. MORRISSEY, and D. D. BIKLE. The effect of  $1,25$  DHCC on calcium accumulation, calcium transport, and calcium binding protein in the presence and absence of cycloheximide. *ibid.* pp 345-347.

4. BIKLE, D. D., R. N. EMPSON, JR., R. H. HERMAN, R. L. MORRISSEY, and D. T. ZOLOCK. The effect of 1,25 dihydroxyvitamin D<sub>3</sub> on the distribution of alkaline phosphatase activity along the chick intestinal villus. *Biochim Biophys Acta* 499: 61-66, 1977.
5. BIKLE, D. D. and H. RASMUSSEN. Ionic Control of 1,25 dihydroxy-vitamin D<sub>3</sub> production by isolated chick renal mitochondria: influence of anions and sucrose. *Biochim Biophys Acta* 538: 127-138, 1978.
6. BIKLE, D. D., D. T. ZOLOCK, R. L. MORRISSEY, and R. H. HERMAN. Independence of 1,25 dihydroxyvitamin D<sub>3</sub> mediated calcium transport from de novo RNA and protein synthesis. *J Biol Chem* 253: 484-488, 1978.
7. CHARLES, A., J. MARTIAL, D. ZOLOCK, R. MORRISSEY, D. BIKLE, and J. BAXTER. 1,25-dihydroxycholecalciferol stimulation of calcium binding protein specific mRNA. (Abstract) *In: Proceedings of the Third Workshop on Vitamin D, Asilomar, CA, Jan 9-13, 1977, p. 57.*
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9. BIKLE, D. D., D. T. ZOLOCK, R. L. MORRISSEY, and R. H. HERMAN. The dissociation of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced CaBP production and alkaline phosphatase activity from calcium transport by actinomycin D and cycloheximide. (Abstract) *ibid.* p. 73.
10. MORRISSEY, R. L., D. T. ZOLOCK, D. D. BIKLE, and P. W. MELLICK. Role of vitamin D dependent calcium binding protein in intestinal calcium absorption. (Abstract) *Fed Proc* 1977.
11. BIKLE, D. D., and H. RASMUSSEN. A biochemical model for the ionic control of 25 hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase. *J Biol Chem* (In press).
12. MORRISSEY, R. L., D. T. ZOLOCK, D. D. BIKLE, R. N. EMPSON, JR., and T. J. BUCCI. Intestinal response to 1 $\alpha$ ,25-dihydroxycholecalciferol: I. RNA polymerase, alkaline phosphatase, calcium and phosphorus uptake in vitro, and in vivo calcium transport and accumulation. *Biochim Biophys Acta* 538: 23-33, 1978.
13. MORRISSEY, R. L., R. N. EMPSON, JR., D. T. ZOLOCK, D. D. BIKLE, and T. J. BUCCI. Intestinal response to 1 $\alpha$ ,25 dihydroxycholecalciferol. II. A timed study of the intracellular localization of calcium binding proteins. *Biochim Biophys Acta* 538: 34-41, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6302	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. ORIGIN INSTR <sup>a</sup>	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102A	3M161102BS02	00	071			
B. CONTRIBUTING							
<del>XXXXXXXXXX</del> CARDS 114f							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) The Metabolic Response of Muscle to Injury, Exercise, and Diet in Health and Disease							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 Clinical Medicine; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PRECEDING			
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		77	
C. TYPE				CURRENT		78	
D. KIND OF AWARD				78		3.4	
E. CUM. AMT.						53	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Department of Medicine Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: 415-561-3600				TELEPHONE: 415-561-4147			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Herman, R. H., COL, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Skeletal Muscle; (U) Myoglobin; (U) Metmyoglobin Reductase; (U) Heatstroke; (U) Oxygen Utilization by Muscle.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23.(U) The acutely injured soldier develops negative nitrogen balance and loses muscle mass. The mechanism for this is unknown. One of the factors that may be involved is myoglobin, a heme protein which transports oxygen within muscle cells. Injury may deplete the enzyme, metmyoglobin reductase, which maintains myoglobin in its functional reduced state. Injured muscle loses myoglobin into the peripheral circulation where it may cause secondary renal damage for unknown reasons. Muscular activity generates increased body heat. If heat generation exceeds heat dissipation, heatstroke (a lethal condition) may be produced. Failure of myoglobin to maintain sufficient intracellular oxygen supplies may result in decreased chemical energy (as adenosine triphosphate) and increased heat production. The myoglobin system and its relation to oxygen utilization and heat production in muscle will be studied.</p> <p>24.(U) Muscle tissue will be obtained from animals, normal human volunteers and patients with a variety of muscle diseases. Metmyoglobin reducing activity will be determined in these muscle tissue samples. Purification of the enzyme, and definitive studies of the properties and characteristics of the purified enzyme will be undertaken. The relationship between metmyoglobin reducing activity, exercise-induced muscle hypertrophy, immobilization-induced muscle atrophy, and recovery of injured muscle will be studied.</p> <p>25.(U) 76 10 - 77 09 Metmyoglobin reductase from bovine heart has been purified repeatedly between 1000- and 1500-fold; the process has yielded materials with specific activities in excess of 40,000. The enzyme so produced is nearly homogeneous on gel electrophoresis. Purified enzyme retains nearly full activity for up to 30 days under appropriate conditions of storage. Limited scale-up will provide sufficient material for definitive study of the properties and characteristics of the enzyme.</p>							



# ABSTRACT

PROJECT NO.	3M161102BS02	Basic Mechanisms of Recovery from Injury
WORK UNIT NO.	071	The Metabolic Response of Muscle to Injury, Exercise, and Diet in Health and Disease

The following investigations have been conducted under this work unit:

STUDY NO. 1    Studies concerning the mechanism which controls the redox state of myoglobin

Previous studies clearly demonstrated the presence of metmyoglobin reducing activity in muscle. The major emphasis this past year has been to purify and characterize this enzyme, metmyoglobin reductase. By utilizing sequential purification procedures the enzyme has been purified between 1000- and 1500-fold, and has yielded specific activities in excess of 40,000 units per mg protein. Conditions which maximize yield and stability have been ascertained. Purification of sufficient enzyme is underway so that the properties and characteristics of the enzyme can be determined.



## BODY OF REPORT

WORK UNIT NO. 071

The Metabolic Response of Muscle to Injury, Exercise, and Diet in Health and Disease

STUDY NO. 1

Studies concerning the mechanism which controls the redox state of myoglobin.

### PROBLEM

Hemoglobin (Hb) of red blood cells and myoglobin (Mb) of red muscle share a number of properties which include reversible oxygenation to form oxyhemoglobin ( $\text{HbO}_2$ ) or oxymyoglobin ( $\text{MbO}_2$ ); or irreversible oxidation to methemoglobin (MetHb) or metmyoglobin (MetMb), respectively. Whether these heme proteins undergo oxygenation or oxidation depends on a number of factors which are complex and not completely understood. Under physiological conditions in vivo, only 2 to 3% of hemoglobin in red blood cells is in the met-form. Several efficient enzymatic systems have been described which continually reduce MetHb, thereby preventing its accumulation to any appreciable extent. The enzymes responsible for this reduction utilize NADH or NADPH, and in some cases require an electron carrier such as methylene blue for in vitro study. By far the most active system requires ferrocyanide ion activation.

Much less attention has been given to the possible existence of similar systems which reduce MetMb. MetMb normally is not thought to be present in muscle in any appreciable quantity despite the greater susceptibility of Mb to oxidation than Hb. It is reasonable to assume that muscle must contain a highly active mechanism for MetMb reduction; otherwise, the continued formation of MetMb would go unopposed. The presence of diaphorases in muscle is well known. However, the existence of a specific MetMb reductase, analogous to MetHb reductase activity in red blood cells, has not been convincingly demonstrated heretofore.

Enzymatic reduction of MetMb by NADH and NADPH dependent mechanisms has been shown by Rossi-Fanelli et al, however, a specific MetMb reductase activity was not found. An enzyme which will reduce metmyoglobin has been described in dolphin muscle. Enzyme activity was demonstrable with either NADH or NADPH at pH 7.0 and required the presence of methylene blue. How this enzyme differs from diaphorase is not clear. Presumed enzymatic reducing activity has also been demonstrated in both intact and ground meat, but without clarification of the mechanism. Furthermore, it has been shown that efficient non-enzymatic MetMb reduction can occur in vitro under suitable circumstances. Moreover, immunologic and electrophoretic studies have shown that ferrocyanide-activated MetHb reductase activity was detectable in several tissues including muscle.

Despite the failure of past investigators to demonstrate convincingly specific enzymatic MetMb reduction, it seemed logical to conclude that if Methb reductase exists in red blood cells, an analogous enzyme for MetMb reduction should exist in muscle. Initial studies (discussed in the 1975 Annual Report pp 101-105) demonstrated the presence of a specific NADH-dependent metmyoglobin reductase in the soluble supernatant fraction of homogenized beef heart. The optimum assay conditions and some of the properties of the enzyme (in the crude system) were established. The next series of studies and experiments were aimed at purifying the enzymatic reducing activity in the supernatant fraction of homogenized beef heart.

Muscle function is impaired in wounded soldiers by direct injury (trauma, muscle wounds, excessive exercise) and/or immobilization of limbs and/or bed rest. In order to facilitate healing and to reverse atrophy of muscle it is important to understand the mechanism involved in exercise-induced hypertrophy and the immobilization-induced atrophy of muscle. It is postulated that myoglobin is involved in these exercise-dependent responses of muscle via its function as an intracellular carrier of oxygen. Since oxygen also oxidizes myoglobin to the met-form it can be logically argued that there must exist a mechanism for the reduction of metmyoglobin. If so, one might expect also that defects in the metmyoglobin reduction system can lead to exercise-induced injury, diminished hypertrophy of muscle during exercise, accelerated atrophy during immobilization, and prolonged recovery after injury.

#### RESULTS AND DISCUSSION OF RESULTS

Highly purified enzyme has been prepared by using the following sequential steps: 1) ammonium sulfate fractionation, 2) carboxymethyl sephadex column chromatography, 3) DEAE cellulose column chromatography, 4) affinity column chromatography with Affi-Gel Blue<sup>(R)</sup>, and, 5) sephacryl S-200 column chromatography. Starting with crude homogenate from beef heart, the enzyme has been purified between 1000- and 1500-fold, yielding specific activities in excess of 40,000 units/mg protein. The purified enzyme retains at least 85% of its initial activity for up to 45 days. Activity during purification and storage is maintained by addition of EDTA to all buffers and dialysis solutions and by rapid concentration of the enzyme solution after each chromatographic step and prior to storage. Enzyme which has been purified by this scheme demonstrates a minor trace of contaminating non-enzyme protein on acrylamide gel electrophoresis. The electrophoretic mobility of the purified metmyoglobin reductase is different than the mobility of the methemoglobin reducing substance of crude RBC hemolysates (presumed methemoglobin reductase) and pig heart diaphorase which has been obtained commercially. We now are preparing sufficient amounts of enzyme necessary to study the properties and characteristics of the enzyme.

### CONCLUSIONS

Previous studies have conclusively demonstrated for the first time the presence of a specific, NADH-dependent, metmyoglobin reductase in the soluble supernatant fraction of homogenized beef heart. Subsequently we have shown that the enzyme can be purified 1000- to 1500-fold and stored for prolonged periods of time without significant loss of activity. Sufficient purification is being performed in order to study the properties and characteristics of the enzyme.

### RECOMMENDATIONS

These studies should be continued.

### PUBLICATIONS

HAGLER, L., R. I. COPPES, Jr., and R. H. HERMAN. Metmyoglobin reductase: Identification of a reduced nicotinamide adenine dinucleotide dependent enzyme from bovine heart which reduces metmyoglobin. (Abstract No. 334) Fed Proc 35: 1423, 1976.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)6.56	
3. DATE PREV. SUMMARY <sup>a</sup>	4. KIND OF SUMMARY <sup>a</sup>	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'S INSTR'M <sup>a</sup>	9. LEVEL OF SUM a. WORK UNIT	
76 10 01	B. Termination	U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO b. SPECIFIC DATA - CONTRACTOR ACCESS	
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3M161102BS02	00	072			
b. SECONDARY	62772A	3S763773S814	00	025			
c. TERTIARY	114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Mathematical and Computer Support of Military Biomedical Research							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
09700 Mathematical and Statistics; 002300 Biochemistry; 021900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
70 02		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER <sup>a</sup> Not applicable				FISCAL YEAR		4.0	
c. TYPE				CURRENT		140	
d. KIND OF AWARD				78		0.0	
e. CUM. AMT.						0	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Department of Information Sciences Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence not applicable				ASSOCIATE INVESTIGATORS			
				NAME: Waibel, K.J., CPT, MS, Harris, D., DAC			
				NAME: L.A. Hopkins, DAC POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Mathematics; (U) Statistics; (U) Research Data; (U) Processing and Analysis; (U) Support of Military Biomedical Research							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. The objective is to provide: (a) a computer facility consistent with functional military research requirement existing within LAIR, (b) technical assistance in interfacing experimental apparatus to data acquisition equipment, (c) a basic library of statistical and general utility programs, (d) assistance to military research project managers in developing mathematical models, designing experiments, formulating testable hypotheses, and interpreting results.</p> <p>24. (U) Consultation is provided in the appropriate and efficient use of available statistical and mathematical programs. Generally, studies are planned to provide valid and thorough statistical analysis for a variety of biomedical research needs. Studies emphasize making analyses readily available to researchers. Presentation of data and analyses are designed so that they may be easily interpreted by investigators who may not have statistical or computer backgrounds. In-house data processing is planned and distributed on basis of need. These systems permit investigators to acquire data automatically and to conduct analyses interactively.</p> <p>25. (U) 76 10 - 77 09. The in-house computer system (a Data General ECLIPSE C/300) is operational and has been providing daily service to all LAIR scientists and their staffs. Eighteen remote terminals have been installed throughout the building for interactive access. On-line plotting capability for the research data has been added to the in-house system. Some general purpose algorithms for common biomedical signal processing such as real-time peak/valley detection have been developed. Data acquisition routines have been developed for instrument interfaces in the areas of biomedical stress measurements and cardio-respiratory data analysis. Consultation in experimental design and statistical analysis has been provided on a continuing basis. This termination reflects consolidation of this work unit with work unit 055.</p>							



# ABSTRACT

PROJECT NO. 3M161102BS02

Basic Mechanisms of Recovery from Injury

WORK UNIT NO. 072

Mathematical and Computer Support of Military Biomedical Research

The following investigations have been conducted under this work unit:

STUDY NO. 1 General mathematical and statistical systems

STUDY NO. 2 Direct mathematical and statistical support of military biomedical research

STUDY NO. 3 Design of distributed information processing facilities

STUDY NO. 5 Biomedical engineering and data acquisition

Mathematical and statistical computing support is available to LAIR investigators from two sources. First, a remote job entry terminal gives access to a CDC 7600 computer at Lawrence Berkeley Laboratory (LBL). Second, the Data General ECLIPSE C/300 in-house computer provides additional, although limited, support. LAIR maintains statistical software on both systems. Statistical consultation is also available on how to use the statistical software as well as how to incorporate statistical tests in experimental design and data analysis. The in-house distributed computer system has been expanded through the procurement of fifteen additional terminals and another minicomputer. Data acquisition on the minicomputer nodes of the network is well underway.

## BODY OF REPORT

WORK UNIT NO. 072

Mathematical and Computer Support  
of Military Biomedical Research

STUDY NO. 1

General mathematical and  
statistical systems

### PROBLEM

Statistics is a collection of methods which allows one to make objective inferences about uncertain data. The term data analysis, however, is used to describe techniques more recently developed in the computer environment. The objectives of computer data analysis have been described as (1) achieving a more specific description of what is loosely known or suspected; (2) finding unanticipated aspects of the data, and to suggest unthought-of-models for the data's summarization and exposure; (3) employing data to assess the adequacy of a contemplated model; (4) providing incentives and guidance for further analysis of the data, and (5) keeping the investigator fully stimulated while he absorbs the feeling of the data and considers what to do next. The objective of this study is to provide the mathematical and statistical computer software necessary for comprehensive data analysis.

It is the philosophy of the Department of Information Sciences that most data analysis procedures may be performed with the use of packaged programs, i.e., stand-alone computer programs specifically designed for data analysis. Such programs are large and do not readily lend themselves to implementation on minicomputers such as the ECLIPSE C/300. Indeed, running large packaged programs on the ECLIPSE would significantly reduce response time to interactive users. Therefore, large packaged programs are maintained at Lawrence Berkeley Laboratory.

### RESULTS AND DISCUSSION OF RESULTS

At Lawrence Berkeley Laboratory, LAIR maintains two statistical packages. The Generalized Research Analysis Statistical System (GRASS) is a package designed to be easy to use for the researcher relatively unfamiliar with computers. GRASS has the capability to produce a variety of descriptive statistics, plots, histograms, and nonparametric tests. GRASS also has a variety of data transformation and data manipulation capabilities. An updated and revised user's manual for the GRASS package was published this year.

In addition to GRASS, LAIR maintains the Biomedical Computer Programs (BMDP) developed at the Health Sciences Computing Facility, University of California, Los Angeles. The BMDP revision implemented at LBL this year includes four new programs:

BMDP2F Two-way frequency tables---empty cells and the identification of departures from independence  
BMDP3F Multiway frequency tables analysis (using log-linear models)  
BMDPAM Description and estimation of missing data  
BMDP9R All possible subsets regression

In addition to the LAIR maintained packages, LBL supports Statistical Package for the Social Sciences (SPSS), an integrated package for data manipulation and analysis. LBL updated SPSS to include several new procedures including MANOVA, a powerful program for analysis of linear models. LBL also maintains a variety of FORTRAN subroutine libraries which may be used to build special purpose programs.

It is acknowledged that packaged programs will not solve all statistical computing problems at LAIR. Often special programs must be written to implement special techniques or data analytic methodologies. The Department of Information Sciences believes that such programs are most easily developed on the ECLIPSE C/300. To facilitate building such programs, the International Mathematical and Statistical Library (IMSL) was acquired and implemented on the ECLIPSE C/300. IMSL contains approximately 150 mathematical and statistical FORTRAN subroutines. Several programs have already been written by using the IMSL building blocks. These programs include basic statistics, principal components analysis, and stepwise regression with residual analysis.

#### CONCLUSIONS

A variety of statistical software is used to perform data analysis at LAIR. Program packages allow an investigator to select which statistical routines are most appropriate without having to master the computational techniques. Lawrence Berkeley Laboratory only supports one statistical package, SPSS. A good remote computing site should provide several such packages, as well as a data base management system which is able to interface directly with statistical packages. The burden to maintain general software should be on the remote computing facility, not on users such as LAIR.

#### RECOMMENDATIONS

It is recommended that the feasibility of obtaining remote batch processing services at another site, which better supports general statistical software, be investigated. Effort to develop specialized statistical software on the ECLIPSE, as applications arise, should continue. BMDP should be updated as new revisions become available.

#### PUBLICATIONS

LAZARUS, H., T. MC CAA, R. TEPLICK, and M. WRENSCH. Generalized Research Analysis Statistical System (Second Edition). Report No. 33. San Francisco, CA: Letterman Army Institute of Research, November 1976



STUDY NO. 2

Direct mathematical and  
statistical support of military  
biomedical research

#### PROBLEM

Efficiency in data collection and analysis is gained by careful planning of experiments, knowledge of appropriate statistical principles and techniques, and a familiarity with available computer software. To attain such a goal, this study provides LAIR investigators with technical support in experimental design and subsequent computer-aided data analysis.

#### RESULTS AND DISCUSSION

Consultation is available, as requested, in experimental design, data analysis and use of mathematical and statistical computer software. Objectives sought in formulating good experimental design are (1) a formal definition of the primary goals of the experiment; (2) employment of a statistical model appropriate to the experimental material which provides unambiguous results, and (3) a design which is feasible within the working conditions of the investigator. Once the data has been collected from the designed experiment, support is available to reduce and interpret the data statistically.

#### CONCLUSIONS AND RECOMMENDATIONS

Appropriate use of statistical methods is essential to accomplishing the scientific research mission at LAIR. Technical mathematical and statistical support centralized in the Department of Information Sciences represents the best way to insure continued available support to all investigators.

#### PUBLICATIONS

PECK, C., and A. HOPKINS. Problems in analyzing pharmacokinetic data. In: Proceedings of the Twenty-second Conference on the Design of Experiments in Army Research Development and Testing, Army Research Office, Report No. 2, (Research Triangle Park, North Carolina, June 1977), pp 11-21.

STUDY NO. 3

Design of distributed information  
processing facilities

#### PROBLEM

There are many reasons why an investigator might choose not to use available computer facilities; inaccessibility because of physical locations; burdensome and tedious data input; lack of software for adequately presenting analyzed experimental data. The objective of

this study is to eliminate these roadblocks to computer usage and maintain a system that provides adequate support to biomedical research activities.

## RESULTS AND DISCUSSION OF THE RESULTS

### Hardware and Facilities

**LBL Computer Services.** The CDC 6600/7600 computer system located at Lawrence Berkeley Laboratory (LBL) computer center, Berkeley, California, continues to provide remote job entry computer support via the CDC 200 user terminal. Unfortunately, this terminal accepts only card input and cannot be hooked up to acquired data from any experimental apparatus.

**Data General ECLIPSE C/300.** This large minicomputer is the host computer for a network of smaller computers and terminals being installed at LAIR. It consists of 128,000 words of memory, two 90 million character disk drives, one industry compatible 9-track tape drive, a 300 line/minute line printer, a 250 card/minute card reader, two 125,000 character floppy disk drives, a high speed paper tape reader, a pen and ink plotter, and communications equipment capable of handling 22 in-house lines and two outside lines. The initial terminal configuration consisted of three CRTs and four teletypes. An additional ten CRTs were acquired through the ADP excess equipment list and an additional five hard copy terminals were procured from Data General Corporation (DGC). Approximately half of these terminals have been installed in selected remote locations throughout the Institute. Thus an investigator need not leave his department area to gain access to a computer. At the terminal he may interactively enter programs and data, and, thereby, bypass any need for transcription of data to punched cards. No other minicomputers have actually been hooked up into the network. However, planning for communications protocols has begun. The capabilities being implemented on each of the individual minicomputers are described in the following paragraphs. The common need being satisfied is that experimental apparatus can be hooked up directly and data acquired in real-time.

**Surgery Data General NOVA 3/12.** This small minicomputer was procured for data acquisition and control of experiments in ophthalmologic testing, thoracic surgery, pulmonary physiology, and anesthesiology. It has been installed and has begun to yield productive results in the past year. Details of the work done on this machine will be described under the appropriate surgery work unit. One notable item is that procedures developed by use of this machine have led to development of a device for ophthalmologic testing on which patents are now pending.

**Military Stress Data General NOVA 3/12.** This is a new minicomputer procured during the past year. It has 32,000 words of memory, two 125,000 character floppy disk drives, a hard copy terminal, 16 channels of analog to digital, and 4 channels of digital to analog conversion capability. It has been installed and functioning in the

Military Stress area for stress data acquisition and analysis. Systems level software has been developed under this study for simplifying access to the analog to digital converter and initiating conversion under control of an external trigger signal. Applications in EEG analysis and the processing of other stress indicators are discussed under the appropriate work unit.

Bioenergetics MODCOMP II. Development of the treadmill automated system (TAS) is continuing. Additional core memory was procured which increased the capacity to 64,000 words. This provides sufficient space for the operating system, TAS software, and application programs.

#### Software Development

Data General ECLIPSE C/300. A network protocol documentation has been acquired from IBM, Digital Equipment Corporation (DEC), and Canadian Bell. Development is beginning on a communications network based on the DECNET protocol. The Terminal Control System (TCS) Plot-10 package has been acquired and modified to run on the ECLIPSE C/300. Through this package a user may specify simply high-level directives which accomplish the plotting of data.

#### Training

Five courses have been conducted by Department of Information Sciences personnel for LAIR scientists and technicians to introduce them to the BASIC programming language available on the central time-sharing computer. At the conclusion of these courses the students have been able to write simple programs in the BASIC program language for their own scientific calculations. This primary training program is continuing and expanding in response to the desire of the LAIR community. An advanced course in data structures was also developed and given.

#### CONCLUSIONS

The in-house computer network continues to grow both in equipment and software capability. Strategically placed terminals and computers make it much easier and more pleasant for investigators to access computing resources. Archiving of data for future analysis is being reduced to a simpler task.

#### RECOMMENDATIONS

Additional terminals and minicomputer nodes must be purchased to provide necessary computing resources to all departments. A network protocol must be developed and implemented to be able to tie all of these together into a functioning network. Extramural support agreements such as LBL must be continued to provide full data base management and statistical support until such time these capabilities can be implemented on the in-house network.



## PUBLICATIONS

None

WORK UNIT 072

STUDY NO. 5

Biomedical engineering  
and data acquisition

## PROBLEM

Rising sophistication of biomedical research activities at LAIR have increased the requirements for biomedical engineering support. The objective of this study is to provide such support as needed by investigators at LAIR, to maintain responsive, state-of-the-art techniques sufficient for future needs, and to collaborate with institute investigators when appropriate.

## RESULTS AND DISCUSSION OF THE RESULTS

**Surgical Recovery Analysis.** This project involves computer storage of the medical records of upwards of 100 surgery patients treated at Letterman Army Medical Center (LAMC), performing various statistical analyses upon these data in order to identify correlation between postoperative treatment and rate of recovery, and generation of mathematical models useful in predicting state of recovery and certain treatment parameters. To date, the special purpose data base for the medical records has been successfully programmed in FORTRAN and implemented on the Data General ECLIPSE C/300. The programming for the statistical analysis is underway for using the International Mathematical Statistical Library as the fundamental source of statistical subroutines. The formulation of the predictive models awaits the results of the statistical analysis.

**Cardiopulmonary Analysis.** This project involves on-line data acquisition of eight (8) cardiopulmonary variables from anesthetized dogs, on-line analysis of several of the variables providing real time feedback to the investigator in the form of analog graphic displays, and extensive off-line analysis of the stored data toward the end of describing the cardiopulmonary effects of various kinds of anesthesia. To date, the on-line data acquisition and analysis programs have been written for implementation on the Data General NOVA 3/12. Partial checkout with the use of simulated data has been successful. Full scale checkout awaits hardware modification of the computer.

**Analog-to-Digital (A-to-D) and Digital-to-Analog (D-to-A) conversion.** Software has been written for A-to-D and D-to-A conversion on the Data General NOVA 3/12 computers within the institute. The software is general purpose, it provides many features for convenience of the using scientist.



Statistical Subprograms. Several general purpose programs have been written, some have used subroutines from the International Mathematical and Statistical Library. These programs will be of general use for a number of computer users within the institute and will encourage direct interaction of the scientist with the computer for analysis of his data.

#### CONCLUSIONS AND RECOMMENDATIONS

Biomedical engineering support and consultation is a recently added dimension of the Department of Information Sciences at LAIR. The primary value of this expertise lies in its provision of an effective interface between computer scientists, mathematicians and statisticians, and biomedical investigators within the institute. This is most important in research requiring on-line data acquisition and analysis, or computer modeling of biological phenomena. Continued development of biomedical engineering within the Department of Information Sciences represents the best way to forge a closer interaction between computer science and biomedical research within LAIR.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6090	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DSSP <sup>a</sup> INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62172A	3S162172A814		00		019	
b. SECONDARY	61102A	3M161102BS02		00		075	
c. TERTIARY	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Investigation of Cell-Free Resuscitating Solutions							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008800 Life Support; 003500 Clinical Medicine; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 03		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING			
b. NUMBER: Not Applicable				FISCAL YEAR		1.5	
c. TYPE:				CURRENT		139	
d. KIND OF AWARD:				78		1.3	
e. CUM. AMT.						149	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
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				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME: DeVenuto, Frank, PhD, DAC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Zuck, Thomas F., LTC, MC			
				NAME:			
				POC: DA			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Acute Resuscitation; (U) Stroma-Free Hemoglobin; (U) Blood Substitute Solutions; (U) Hemorrhagic Shock							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Precede each with Security Classification Code)							
<p>23. (U) Hemoglobin, free of cell constituents, may be an ideal resuscitation fluid for the severely wounded soldier. It is capable of in vivo on-loading and off-loading oxygen with sufficient efficiency to maintain normal oxygen consumption in experimental animals rendered virtually free of circulating red cells. Solutions of hemoglobin are superior to electrolyte solutions in resuscitation of animals following acute hemorrhagic shock. Hemoglobin can be stored for extended periods. The object of these studies is to evaluate the practicality of the hemoglobin solution as a resuscitation fluid for military use.</p> <p>24. (U) Hemoglobin, prepared by crystallization, as the protein base for a cell-free resuscitation solution is being evaluated in animal models for its effect on critical organ function and maintenance of morphological integrity. Storage stability under various conditions is being studied; methemoglobin formation is used as an index of deterioration. Optimal concentrations of electrolytes and supplemental protein for improved in vivo function are being determined.</p> <p>25. (U) 76 10 - 77 09 Total and partial exchange transfusion of hemoglobin solutions in rodents have shown superior maintenance of vital signs, improved survival and superior preservation of liver, kidney, and brain morphologic integrity compared with control rodents exchanged with albumin solutions. When glucose is used as a stabilizer, hemoglobin solutions have been lyophilized, stored, and reconstituted with only negligible methemoglobin formation. Large scale preparation of hemoglobin has been accomplished in cooperation with a pharmaceutical company.</p>							

<sup>a</sup> Available to contractors upon originator's approval.

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

# ABSTRACT

PROJECT NO. 3M161102BS02 Basic Mechanisms of Recovery from Injury  
WORK UNIT NO. 075 Investigation of Cell-Free Resuscitating Solutions

The development and evaluation of the effectiveness of hemoglobin solutions as a resuscitation and oxygen carrying blood substitute have been continued. Further in vivo studies have shown that hemoglobin-transfused rodents maintain an effective colloidal osmotic pressure (oncotic pressure, 17-21 torr) and a constant difference in oxygen content between arterial and venous blood (3-5 ml%) during and after transfusion. Morphological and biochemical studies demonstrate that the structural integrity of the liver, kidney, and brain is maintained at early time intervals after hemoglobin transfusion. Functional aspects of the hemoglobin molecule have been investigated with attempts to increase the oxygen release capacity which is important in the off-load of oxygen to tissues following transfusion.

If the logistic requirements of combat situations are to be fulfilled in the transfusion of mass casualties, this blood substitute must be available in large quantities and must be stable for long periods of time. The production technique for purifying hemoglobin from outdated lysed human red cells by crystallization has been successfully adapted to large scale preparation, and the mass production capacity has been proven. Data on long-term storage indicate that hemoglobin solutions stored at -20 C for 2 years do not show any significant alteration in the stability and function of hemoglobin. At 4 C, they remain unchanged for a period of 12 months. With the use of 3% glucose as a stabilizer, solutions of hemoglobin have been successfully lyophilized, without significant metabolic or functional alteration. Samples of lyophilized hemoglobin have been kept at room temperature and analysis after 3 months shows that no significant deterioration has occurred. Lyophilized hemoglobin can be reconstituted in solution by addition of sterile water.

Hemoglobin solutions appear to have the potential to become an ideal blood substitute and to provide a suitable resuscitation fluid for the severely wounded soldier. They are uniquely suited to fulfill the supply, storage, and transportation requirements for field use in combat situations.



## BODY OF REPORT

WORK UNIT NO. 075

Investigation of Cell-Free Resuscitating  
Solutions

### PROBLEM

Resuscitating solutions, to be effective, must meet stringent requirements. As blood substitutes they must provide the basis for a life support system and for restoring vital functions in mass casualties, and they must be uniquely suited to fulfill the supply, storage, and transportation requirements for field use in combat situations.

Plasma, dextran, albumin, and other preparations have been used and, although they appear to be effective as plasma expanders, they are of limited use as blood substitutes since they do not carry oxygen. As a resuscitating fluid, blood has a limited storage life and requires typing and cross-matching prior to use.

Great advantages may be gained by the development of a solution capable of transporting oxygen, maintaining oncotic pressure, and being readily available when massive clinical transfusions are required.

In most civilian settings in this country, the transfusion requirements associated with massive trauma can be met with conventionally stored blood and its components. However, military needs frequently demand massive fluid support in areas remote from supply sources; these needs present unique and difficult problems in storage and transportation of these fluids. The inability to predict when modest transfusion requirements may suddenly increase further complicates fluid therapy logistics. The ability to stockpile a stable protein solution capable of carrying and exchanging oxygen would avoid many of these difficulties.

Hemoglobin is a protein which has such potential. Hemoglobin can transport and exchange oxygen, has oncotic activity, can be prepared from outdated blood, does not require typing and cross-matching, can be stored for long periods of time under sterile conditions, has low viscosity, and is thought to have a low allergic potential. However, it is imperative that if hemoglobin is used as a blood substitute, it must be free of any stromal particle, stromal lipid, or other soluble and insoluble cell components which have been implicated in adverse effects on kidney function and on coagulation factors. Hemoglobin has the potential to become an important blood substitute and may be a suitable resuscitation for the severely wounded soldier. The problem of developing an effective blood substitute is pertinent not only to the military for its combat casualties, but also to civilian agencies for casualties in accidents and mass disasters.

## RESULTS AND DISCUSSION OF RESULTS

Methods advocated for the preparation of hemoglobin solution require hemolysis of erythrocytes, high speed centrifugation, and filtration through a 0.2  $\mu$  millipore filter to remove stromal particles. With these techniques, however, there remains the possibility of contamination with soluble components of the red cell membrane. As previously reported, a rapid, simple, and reproducible method has been developed in our laboratory for the preparation of hemoglobin solution. The method, based on the crystallization procedure, yields hemoglobin free of soluble and insoluble red blood cell membrane components. The hemoglobin thus prepared is also free of antigenic substances and does not affect conventional coagulation tests. The effectiveness, as a transfusion substitute for blood, of hemoglobin prepared by crystallization has been demonstrated in total and partial blood replacement studies in laboratory animals, as reported recently.

**Large Scale Preparation.** In the preparation of hemoglobin by the crystallization procedure, high speed centrifugation for the sedimentation of small particles is not necessary, as all centrifugations are at low speed and adaptable to batch processing. This is important for large scale preparation to process high volumes of outdated blood to yield quantities of hemoglobin solutions necessary for clinical transfusions. Our laboratory has served as a consultant to Hyland Division, Travenol Laboratories, Costa Mesa, California (Dr. W.R. Thomas, Director, Therapeutic Research). That company has allocated resources for large scale preparation of hemoglobin. The production technique (i.e., by crystallization) for preparation of hemoglobin from outdated lysed human red cells has been adapted and the mass production capacity has been proven. We tested 20 different preparations made at Hyland to ascertain that our specifications were met. Recently, 4 gallons of hemoglobin solution were obtained for transfusion studies in higher animals. Investigators at Hyland have also determined certain *in vitro* and *in vivo* characteristics of hemoglobin and have confirmed our published data.

**Oncotic Pressure of Hemoglobin Solution.** It has been postulated that hemoglobin solutions exert osmotic pressure because hemoglobin is a high molecular weight (68,000) protein not able to pass through the pores of a semi-permeable membrane. The dissolved proteins of the plasma are responsible for the osmotic pressure that develops at the capillary membrane and this osmotic pressure is called "colloid osmotic pressure" or "oncotic pressure."

The oncotic pressure exerted by the hemoglobin solution has been determined quantitatively since it is important that an effective oncotic pressure is maintained *in vivo* during and after transfusion. The oncotic pressure of human plasma and hemoglobin solution has been determined as a function of protein concentration and the results have shown no significant differences between these 2 fluids at the several protein concentrations investigated (0.5-8 g/dl).

In studies in vivo, groups of rats were transfused with a 7 g/dl hemoglobin (experimental, n=4) or a 7 g/dl albumin solution (control, n=4) to 25% of the initial packed cell volume. Immediately after transfusion, both groups showed similar "plasma" oncotic pressure of 20-21 torr, higher than that observed in normal rat plasma (17-18 torr at serum protein concentrations of 5.9 g/dl). During the first 24 hours after transfusion, oncotic pressure values of 19-20 torr were determined in the control, and values of 15-17 torr were obtained in the experimental group, reflecting the different intravascular life of albumin and hemoglobin. After 6 to 8 days, the oncotic pressure returned to normal values (17-18 torr). Plasma protein electrophoresis in experimental and control groups demonstrated a rapid replacement of proteins. By 6 hours the intravascular albumin of the experimental group had been replenished to 57% and, by 24 hours, to 83% of the preinfusion concentration. The data demonstrate that the rapid loss of infused free hemoglobin may pose more of a theoretical than a practical clinical limitation from the standpoint of maintaining oncotic pressure.

**Morphological Studies of Organs.** Investigations on possible effects of hemoglobin solution on the histology and ultrastructure of cells of transfused rats have been continued. Livers, kidneys, and brains of rats transfused to a 75% blood replacement with hemoglobin or albumin solution were removed at 1, 5, 12, and 24 hours, and also at 2 months after transfusion. The organs were fixed and sections cut for light and electron microscopic examination. The results show that livers of rats transfused with hemoglobin appear normal at 1 and 5 hours following completion of exchange. In contrast, livers of animals transfused with albumin demonstrate marked centrilobular vacuolization and mitochondrial abnormalities. At 12 and 24 hours, livers from experimental animals show randomly distributed focal areas of centrilobular abnormality, whereas at these time periods, control rats show small foci of hepatocellular necrosis. By 2 months the architecture of the liver cells is normal in all animals.

Kidneys of the experimental animals show intracellular droplets at 24 hours after transfusion. Intratubular material, presumably hemoglobin, appears in greatest quantity at one hour and a small proportion of collecting and distal tubules still contain hemoglobin at 24 hours. Albumin-exchanged animals demonstrate little increase in proximal tubular cell droplets and scant intratubular material.

Cerebral cortex, hippocampus, and cerebellum appear normal at all time periods studied in all animal groups.

These results suggest that during the early time periods following exchange transfusion, hemoglobin protects the liver from hypoxia, presumably by its ability to transport and off-load oxygen. The rapid clearance in the rat (one-half disappearance time of 3.5 hours) of the hemoglobin may explain the randomly observed centrilobular alterations at 12 and 24 hours after transfusion. The presence of hemoglobin in



the tubular lumens represents hemoglobin being excreted through urine. Hemoglobin does not appear to affect renal tubular epithelial ultra-structure adversely.

**Further in vivo Evaluation of Hemoglobin Solution.** Preliminary studies have been done to monitor in vivo physiological parameters of animals transfused to 95% blood replacement with hemoglobin or albumin or other resuscitating solutions. The results indicate that to maintain constant central venous pressure, replacement of one volume of blood requires 1.6 volumes of plasmanate or 4.1 volumes of Ringer's lactate, whereas when hemoglobin or albumin solution is used, a 1:1 volume ratio is sufficient. Furthermore, in hemoglobin-transfused animals, the heart rate and the body temperature remain constant during transfusion, whereas in albumin-transfused animals, the heart rate falls from a pre-transfusion level of 420 to a post-transfusion value (2% hematocrit) of 200, and the body temperature shows a corresponding decrease from 35 to 32 C (95 to 89 F).

The arterial and venous oxygen contents were measured during and up to 3 hours after transfusion in animals exchanged with hemoglobin solution to 95% blood replacement. The arteriovenous oxygen contents declined during transfusion but remained fairly constant in the post-transfusion period. The A-V<sub>O<sub>2</sub></sub> difference was maintained during the time investigated, which indicates that the extraction of oxygen from the circulating arterial fluid continues even when 95% of blood is replaced by the hemoglobin solution.

Blood volume determinations were made in groups of rats during transfusion and at 1, 3, 6, 18, 24, and 48 hours after transfusion to 75% blood replacement with hemoglobin or albumin solution. The results show that in the experimental animals a gradual loss of blood volume occurs during the first 6 hours after completion of transfusion. The blood volume returns to normal pretransfusion levels at 24 hours following transfusion. In control animals the blood volume loss during the 6 hours following transfusion is small, and at 24 hours normal blood volumes are observed. These differences possibly can be attributed to the different intravascular life of hemoglobin and albumin.

In other experiments, rats have been transfused to 90% blood replacement with a solution containing a mixture of hemoglobin prepared by crystallization (7%) and albumin (5%). The animals appear to benefit from this mixture since all the animals survived without additional transfusion, whereas control animals died at 5 hours following 90% blood replacement with hemoglobin solution of 7 g/dl nonsupplemented with albumin. Preliminary data show that in animals transfused with hemoglobin-albumin mixtures, the loss of blood volume is greatly minimized and the oncotic pressure is higher than normal values. After transfusion, the plasma oncotic pressure shows values of 34 torr; it returns to normal levels at one day after transfusion. These findings need further clarification by additional experiments.

Functional Aspects of Hemoglobin. It is important to have knowledge of the oxygen dissociation curve and of the several factors which may influence the oxygen affinity to the hemoglobin molecule, since hemoglobin, as prepared by the crystallization procedure, is stripped of the organic phosphate compounds present in the red cell and which have been reported to affect oxygen-hemoglobin interactions. To improve the oxygen release capacity of hemoglobin, attempts have been made to reduce oxygen affinity. The results have shown that oxygen affinity decreases slightly by addition of "diatrizoate sodium" and considerably by addition of "acetotrizoate sodium" to the hemoglobin solution. Since these compounds could be clinically unacceptable, organic phosphates such as 2,3 diphosphoglycerate, adenosine triphosphate, and pyridoxal-5'-phosphate, have been studied. The data show that these compounds interact with the hemoglobin molecule and influence the  $P_{50}$ , improving the oxygen release capacity. This effect is dependent on the concentration of the organic phosphates and is observed, at physiological levels, only in hypotonic media. In isotonic solution, no effect is obtained unless the concentration of the phosphate compounds is 50 to 100 times greater than the physiological values. In vivo studies show that when organic phosphate is added to the hemoglobin solution infused into rats, the  $P_{50}$  of the intravascular fluid is not affected by their presence. The influence of salts and of pH on the oxygen dissociation curve of hemoglobin has also been studied and the Bohr effect plotted. We obtained data similar to those reported in the literature. Other studies have demonstrated that residual glycolytic enzymatic activity is associated with hemoglobin prepared by a variety of procedures, including crystallization. The implication of this enzymatic activity on the structural and functional integrity of the hemoglobin molecule is being investigated.

Stability of Hemoglobin. If logistic requirements of combat situations are to be fulfilled in the transfusion of mass casualties, the hemoglobin solution, used as a blood substitute, must be available in large quantities and must be stable for long period of time. The mass production capability of the preparation procedure of hemoglobin has been proven, as described before. Data on storage indicate that hemoglobin is stable for a long time. Hemoglobin solutions, prepared by crystallization and maintained in blood bags under sterile conditions, were stored at refrigerator (4 C) or freezer temperature (-20 C). At several intervals, methemoglobin content,  $P_{50}$ , n values, osmolality, oxygen dissociation curve, oxygen capacity, Na, K, and pH were analyzed. Solutions maintained at -20 C do not demonstrate any alteration in these parameters after a storage time of 2 years. Solutions maintained at 4 C do not show any deterioration for a period of 12 months. After 12 months and especially after 18 months, deteriorations are evident in these solutions, as demonstrated by increases in methemoglobin content and decreases in the  $P_{50}$  value.

Although storage of this blood substitute for such an extended time at refrigerator temperatures represents a great improvement over the

limited storage time of blood, it would be desirable if hemoglobin could be maintained at room temperature. In solution under sterile conditions and at room temperature, hemoglobin is transformed to methemoglobin in relatively short periods of time. However, through the process of lyophilization, solutions of hemoglobin have been reduced to a powdered form in the presence of glucose, used as a stabilizer to prevent formation of methemoglobin during the process. Lyophilization has been done under sterile conditions. The powdered hemoglobin thus obtained can be reconstituted in solution by addition of distilled sterile water; a solution ready for transfusion when needed is obtained. Samples of lyophilized hemoglobin have been kept at room temperature; analysis after 3 months shows that no significant deterioration has occurred.

#### CONCLUSIONS

Continued efforts to evaluate the practicality of the hemoglobin solution as a resuscitation and oxygen-carrying blood substitute indicate that hemoglobin, free of cell membrane constituents, can provide the basis for suitable resuscitation fluid for the severely wounded soldier. The mass production capacity of the hemoglobin obtained by the crystallization procedure from outdated lysed human red cells has been proven. The hemoglobin solution is stable for long periods of time; this fulfills the logistic requirements for supply, storage, and transport when massive fluid support is needed in the transfusion of military combat casualties as well as civilian casualties. The data obtained in vivo on the plasma oncotic pressure demonstrate that the rapid loss of infused free hemoglobin may pose more of a theoretical than a practical clinical limitation from the standpoint of maintaining an effective oncotic pressure. As a blood substitute, hemoglobin appears to be beneficial in restoring and maintaining vital signs and does not appear to cause adverse effects, morphologically, to liver, kidney, or brain cells.

#### RECOMMENDATIONS

Hemoglobin, available now in large quantities, should be used for clinical studies in higher animals. These studies will allow us to monitor, with a high degree of accuracy, a larger range of important physiological, biochemical, and hematological parameters. Investigations on the morphology and function of organs of animals transfused with hemoglobin solution should be continued in order to exclude completely any potential side effects which might occur during and after transfusion. Increasing efforts should be focused on the functional aspects of the hemoglobin molecule, namely, the increase of oxygen release capacity of hemoglobin (i.e., higher  $P_{50}$ ), by a direct combination of hemoglobin with an organic phosphate compound such as pyridoxal-5'-phosphate. The removal of the residual enzymatic activity and its possible effect on the structural and functional integrity of the hemoglobin molecule should be further investigated. Also, consideration should be given to increasing the retention time of hemoglobin in the plasma after



transfusion. The development of hemoglobin from sources other than human blood should be explored for their potential as blood substitutes. The efforts in preparing hemoglobin in a powdered form to be maintained for extended periods at room temperature need to be intensified since this achievement is promising and important for solving supply, storage, transportation, and other logistics problems.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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a. PRIMARY	62172A	3M162172A810		00		001	
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23. (U) Linea corporis (body ringworm) has produced unacceptable non-effectiveness of combat troops in previous military conflicts. This problem may be anticipated within the D + 15-30 time frame of possible future conflicts unless adequate preventive measures are found. The objectives of this study are to investigate the mechanism of initiation of infection, the immunologic response, and therapeutic modalities in animal and human models.							
24. (U) Initiation of infection in animals is relevant to the discovery of improved prophylaxis; therefore the approach will include studies: (a) in the sequential histopathology by light and electron microscopy, (b) on influence of changes in local skin environment, and (c) on relative infectivity of fungal particles. Further defining the immune response to fungal infections by techniques such as chemotaxis, lymphocyte transformation, skin testing, and antibody assays, is designed to yield insight into the mechanism of induction of protective immunity. Prevention of infection is the major goal; determining how to decrease the severity of disease by drug therapy or alterations of the micro-environment will be steps toward that goal.							
25. (U) 76 10 - 77 09. Under controlled conditions with experimental infections on the skin of guinea pigs, fungal morphology, microanatomical routes of invasion, and sites/rates of proliferation were defined. Studies of protective immunity revealed that the degree of immunity was not related to repeated infections or by the severity of the first infections. Vaccination did not always confer immunity; some skin test positive animals were not protected from infection. A new micromethod to assay the effects of antifungal agents was developed. Deferoxamine B (an iron-chelator) was determined to be fungicidal under defined conditions.							

\*Available to contractors upon originator's approval.

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# ABSTRACT

PROJECT NO. 3M762772A810 Other Tropical Medicine  
WORK UNIT NO. 001 Prevention of Fungal Infections of  
the Soldiers Skin

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Host parasite relationships in dermatophyte infections
- EX-1 A visual micromethod for assay of fungal growth
  - EX-2 Effect of iron-deprivation on fungal growth
- STUDY NO. 3 Immunology of fungal infection
- EX-1 A non-occluded model for studying dermatophytosis in guinea pigs
  - EX-2 Hairless dogs as models for dermatophyte infections
  - EX-3 Immunization with trichophytin
  - EX-4 Local immunity
  - EX-5 Comparison of the degree of immunity with normal or severe infections, or with one or several reinfections

STUDY NO. 1 This is a microscopic study of when, where, and how dermatophytes invade skin. Techniques have been developed and applied to non-occluded experimental Trichophyton mentagrophytes infections in guinea pigs. Microscopic observations have revealed that (1) spore germination on the stratum corneum surface is rare or non-existent; (2) entrance to the stratum corneum and hair follicle seems to be via the opening of the hair follicle, not through the intact surface of the stratum corneum; and (3) the lowest portion of the stratum corneum (stratum lucidum) is the pathway selected by the peripherally spreading fungus. Further studies will be done to determine how this pattern differs for occluded or abraded skin.

EX-1 A micromethod was developed to assay fungal growth.

EX-2 A micro method was developed and it was determined that deprivation of iron by the iron-chelating agent, deferoxamine mesylate, inhibits fungal growth at 5 mg/ml.



STUDY NO. 3 There is not always a positive correlation between immunity and conversion to positive skin test responses. Increased or complete immunity could not be induced by creating more severe lesions or by repeated infections. It has been found that complete immunity exists only when infections are attempted at a hairless, previously infected site.

EX-1 An economical method of producing fungal infections in guinea pigs was developed.

EX-2 Hairless dogs were infected. They demonstrated self-limited fungal infections. They are superior to guinea pigs in some aspects for testing topical antifungal agents.

EX-3 There is not always a positive correlation between immunity and conversion to positive skin test responses.

EX-4 Complete immunity exists only when infections are attempted at a hairless, previously infected site.

EX-5 Increased or complete immunity could not be induced by more severe lesions or repeated infections.

## BODY OF REPORT

WORK UNIT NO. 001

Prevention of Fungal Infections of the  
Soldiers Skin

STUDY NO. 1

Host parasite relationships in  
dermatophyte infections

### PROBLEM

In the combat soldiers' environment, disease produced by Trichophyton mentagrophytes may increase in severity and result in epidemic disabling body ringworm. By microvisual techniques, we investigated when and where in the skin the fungus invades. We hope to determine the fungal forms and their exact microanatomic pathways of invasion under various local skin conditions. These patterns may indicate inherent strengths or weaknesses of the host and parasite species as they interact, which may lead to improved prophylactic or therapeutic measures or agents.

### RESULTS AND DISCUSSION OF RESULTS

Histopathologic and ultrastructural studies of host-parasite relationships were begun during the past year. The prerequisite techniques of biopsy, light microscopy, scanning electron microscopy, and transmission electron microscopy were developed. These techniques were used to study the LAIR standardized non-occluded guinea pig infection with Trichophyton mentagrophytes. Each day for 10 days (then every other day for 20 days), three infected guinea pigs were sacrificed and subjected to total lesion excision biopsy. Examination of specimens by all 3 microscopic methods supports the following conclusions:

(1) germination of spores applied to the stratum corneum surface is rare or non-existent in this model, even though infection occurs consistently; (2) entrance to the stratum corneum and hair follicle seems to be via the opening of the hair follicle, not through surface penetration of the intact stratum corneum; and (3) the lowest portion of the stratum corneum (stratum luicum) is the pathway for peripheral spread of the fungus.

A new technique was developed for scanning electron microscopy. Biopsied skin was fixed prior to cross-sectioning with a razor blade; after critical point drying and metal coating of the specimen, the exposed cross-sectional edge was examined. This technique revealed unique microvisual information about interactions of this host and parasite.

(1) Considerable mechanical pressure is probably exerted by the invading hyphae, as evidenced by the compliant circular distortion of the surrounding stratum corneum laminae, and also by the marked rigging of the hair cuticle, caused by hyphae tunneling beneath it. (2) hyphae proceeding down a hair follicle do not stay exclusively within the space surrounding the hair shaft, or within the follicle

wall, as proposed by two prior investigators; the hyphae can weave in and out of both regions. Open furrows produced by hyphae are a striking feature on the surface of the hair shaft and on the opposed structure (the inner surface of the internal root sheath). (3) "Cuticular lifting" or wedging of the hyphae beneath the free edge of a cuticular plate was observed; direct penetration of the cuticular plates was also observed. (4) Arthrospore formation of hyphae in the space between the hair shaft and follicle wall was observed; it is similar to the process described at LAIR for hyphae grown in vitro under CO<sub>2</sub>. (5) The scanning electron microscope permits one to see graphically the massive amount of fungus present in follicles as compared to the stratum corneum.

Preliminary examinations by transmission electron microscopy revealed regions of keratin adjacent to hyphal walls (or tips) that appear eroded and filled with fragmented fibrillated material. The appearance is consistent with an enzymatic lytic process.

In other experiments with quantitated infections, we have established that (compared to unmanipulated skin) the number of spores required for infection decreases if infection is initiated with occlusion or abrasion. However, we have also demonstrated that continuous occlusion produces an abbreviated infection compared to a short period of occlusion. Microscopic examination of alterations in host or parasite that attend these conditions can help to determine the mechanisms underlying the change in spore infectivity and pathogenicity.

#### CONCLUSION

The experimental dermatophyte infection model that we have examined sequentially has shown a definite invasion pattern; including subsurface germination and peripheral expansion through the lowest layers of the stratum corneum. Scanning electron microscopy has yielded unique views of interaction between a dermatophyte and its host. Most of these findings either have not been observed or have not been reported.

#### RECOMMENDATIONS

Experiments should be conducted to examine microscopically the particular activity of T. mentagrophytes in infections induced by various periods of occlusion and on abraded skin.

#### PUBLICATIONS

None



STUDY NO. 3

Immunology of fungal infection

EX-1

A visual micromethod for assay of  
fungal growth

#### PROBLEM

Present techniques for assaying antifungal agents are expensive, laborious, and require large amounts of test agents. Therefore, we needed to develop a microassay that overcame these limitations.

#### RESULTS AND DISCUSSION OF RESULTS

A visual micromethod for measuring effects on germination and growth of fungi was developed. With this micromethod, spores are suspended in 0.2% agarose. A 2  $\mu$ l droplet of this spore-agarose suspension (containing 600 spores) is placed in a well of a microtiter plate. Culture medium with or without test agents is then added. The spore-agarose droplets are viewed at 18 hours with an inverted microscope for evidence of germination. Fungal growth is assayed at 24 and 48 hours by measuring hyphal lengths or determining the number of squares occupied with hyphae in a 100 square grid placed in the microscope eyepiece.

The antifungal agent, griseofulvin, and the fungus, Trichophyton mentagrophytes were used as the materials to compare the micromethod with a standard assay based on dry mycelial weight. The micromethod was more sensitive than the weight method in detecting the minimum inhibitory concentration of griseofulvin (0.18 and 0.35  $\mu$ l/ml, respectively). At higher concentrations of griseofulvin (22.5  $\mu$ g/ml), the micromethod measured minimal fungal growth that was undetectable on a weight basis. Direct visualization of organisms by the micromethod determined that griseofulvin does not change the number of spores forming germ tubes. Progressively severe alterations in fungal morphology occurred as the concentration of griseofulvin was increased from 0.09 to 22.5  $\mu$ g/ml.

#### CONCLUSIONS

A micromethod, developed for assaying antifungal effects of test agents, is sensitive, and it saves about 80% of the cost of other antifungal assays.

#### RECOMMENDATIONS

The micromethod is economical and should be used to assay soluble or slightly soluble antifungal agents.

#### PUBLICATIONS

1. KERBS, S., R. HUTTON, and J. HOLLISTER. A visual micromethod for assay of fungal growth. Can J Microbiol (in press)

EX-2

Effect of iron-deprivation on  
fungal growth

#### PROBLEM

New topical antifungal agents need to be found which will be safe for human use. There are a number of iron-chelating agents that are now being used in humans for iron-poisoning and iron related diseases. We investigated the usefulness of one iron-chelating compound, deferoxamine mesylate, on its ability to inhibit fungal growth by deprivation of iron.

#### RESULTS AND DISCUSSION OF RESULTS

Deferoxamine mesylate (Desferal<sup>R</sup>), an iron chelator, inhibited germ tube formation and growth of Trichophyton mentagrophytes in a microculture assay. A 50% reduction of germination required 5 mg/ml deferoxamine and a 50% reduction of growth required 2 mg/ml. Iron (133 µg ml) reversed the inhibition of growth produced by incubating spores with 5 mg/ml deferoxamine, providing iron was added before 72 hours incubation. Deferoxamine at 100 mg/ml decreased viability of activated spores incubated for 3 days at 30 C, but did not decrease viability of spores incubated for 3 days at 4 C.

The growth inhibitory effects of deferoxamine and transferrin were compared. Transferrin was inhibitory at low molarities (0.001 to 0.1 mM), while deferoxamine was inhibitory only at higher molarities (greater than 1 mM). It (0.05 mM) also reversed the inhibition expected with 0.05 mM transferrin.

#### CONCLUSIONS

These findings indicate that deferoxamine mesylate (Desferal<sup>R</sup>) and transferrin deprive T. mentagrophytes of nutritional iron which results in inhibition of growth. Since low concentrations of Desferal<sup>R</sup> can also promote growth (in the presence of transferrin), it will probably not be suitable for preventing fungal infections.

#### RECOMMENDATIONS

Depriving fungi of nutritional iron should be investigated for its potential usefulness in preventing or diminishing fungal infections in soldiers.

#### PUBLICATIONS

1. KERBS, S., R. HUTTON, M. LANCASTER, and K. JESRANI. Deferoxamine inhibition of Trichophyton mentagrophytes. (Abstract) Am Soc Microbiol 1977. (New Orleans, LA, 8-13 May 1977)
2. KERBS, S., R. HUTTON, M. LANCASTER, and K. JESRANI. Effect of deferoxamine mesylate on Trichophyton mentagrophytes. Submitted for publication.

STUDY NO. 3

Immunology of fungal infection

LX-1

A non-occluded model for studying dermatophytosis in guinea pigs

#### PROBLEM

The present technique for inducing fungal infection in guinea pigs is laborious. We needed to find a simplified reproducible method.

#### RESULTS AND DISCUSSION OF RESULTS

A non-occluded model for studying dermatophytosis in guinea pigs was developed. This model is more economical in time and money than the previous model which initiated infections with occlusion. The guinea pig does not need to be epilated or bandaged. In the non-occluded model, infections are initiated by placing 1,000,000 spores on 13 mm<sup>2</sup> area of the guinea pigs' furry back. Standard size lesions develop; the lesions follow a predictable course.

#### CONCLUSIONS

Guinea pigs can be infected by placing 1,000,000 spores on their skin without occlusion, shaving, or injury to the skin.

#### RECOMMENDATIONS

This simplified technique should be used as applicable.

#### PUBLICATIONS

None

LX-2

hairless dogs as models for dermatophyte infections

#### PROBLEM

We would like to find an animal which would have long-lasting chronic fungal infections. This animal could be used to investigate the immunological response in fungal diseases and would be particularly useful for testing antifungal agents. We investigated the potential



for developing chronic infections in Mexican hairless dogs which were raised at LAIR.

#### RESULTS AND DISCUSSION OF RESULTS

In search for a model for chronic dermatophytosis, we tested Mexican hairless dogs and compared their infections to haired dogs. Lesions of hairless vs. haired dogs, although morphologically different, were similar in development, size, and duration.

#### CONCLUSIONS

Hairless dogs were not chronically infected. The lesions in dogs are longer-lasting (50 days) and allow more time to discriminate between the effectiveness of antifungal agents than is allowed by the shorter lasting lesions we have seen in guinea pigs (14 days).

#### RECOMMENDATIONS

Mexican hairless dogs may be useful in testing topical antifungal agents and tests should continue.

#### PUBLICATIONS

1. HUTTON, R., and S. KERBS. Experimental Trichophyton mentagrophytes infection in hairless dogs. Lab Animal Sci (in press).

EX-3

Immunization with trichophytin

#### PROBLEM

A new lot of fungal antigen was prepared at LAIR. This lot was tested in guinea pigs to determine if it could confer immunity to dermatophytosis.

#### RESULTS AND DISCUSSION OF RESULTS

This antigen converted guinea pigs to positive skin test responses upon (1, 2) injections. However, although animals became skin test positive they did not demonstrate any increased resistance to infection. This antigen also did not elicit a cutaneous basophilic hypersensitivity response which could be elicited with some of the previous fungal antigen lots.

#### CONCLUSIONS

The presence of a positive skin test response does not always mean the subject is immune to infection.

#### RECOMMENDATIONS

Each lot of antigen will have to be tested for its immunogenicity. Ideally, we would like to have an in vitro test for immunity.

#### PUBLICATIONS

None

EX-4

Local immunity

#### PROBLEM

The basic question of the type of immunity that is induced by a fungal infection needs to be resolved before human studies are further explored. There are various reports on the type of immunity that exists after a primary infection. Investigators have stated that complete immunity over entire body exists, that complete immunity only at original site of infection exists, or that only a partial immunity exists to reinfection.

#### RESULTS AND DISCUSSION OF RESULTS

We investigated the degree of immunity produced with T. mentagrophytes var. granulare ATCC 18748. Guinea pigs were inoculated with fungus and then reinfected both at the site of the first infection and at a previously non-infected site. This was done at three time intervals, 1) when infection sites were still bald, 2) when infection sites had more than normal amounts of hair, and 3) when infection sites appeared normal. We found that all previously non-infected sites developed secondary lesions. Sites that were previously infected did not develop lesions if hair was absent, but did develop lesions once hair had regrown at the site.

#### CONCLUSIONS

Partial immunity follows a primary fungal infection. The original site of infection is resistant to reinfection if that area is still hairless, but it is not resistant to reinfection if hair has grown back.

#### RECOMMENDATIONS

None

#### PUBLICATIONS

None

Comparison of the degree of  
immunity with normal or severe  
infections, or with one or  
several reinfections

PROBLEM

Humans and animals develop only a partial immunity to fungal infections. We need to find a method which will confer complete immunity. One suggestion for such a method is to have a severe primary infection. Another suggestion for such a method is to reinfect animals several times. Both methods were investigated.

RESULTS AND DISCUSSION OF RESULTS

The degree of immunity induced by a normal small infection was compared to that induced by a severe infection. Guinea pigs were divided into 2 groups. One group of animals was inoculated at one site with fungal spores. The other group was inoculated at 20 sites with spores so that lesions covered their entire body. The degree of immunity produced was assessed by the severity of secondary lesions. Both groups of animals had equally severe lesions, and therefore a small infection produced the same degree of protection as numerous infections. We also investigated the degree of immunity following several successive reinfections. Guinea pigs were infected with fungal spores, allowed to heal, and then reinfected. This was repeated four times. The fourth infection was similar to the second infection in the degree of severity. Therefore animals did not develop increased resistance by repeated infections.

CONCLUSIONS

We could not induce complete immunity in guinea pigs by increasing the severity of the primary infection nor by reinfecting the animals several times.

RECOMMENDATIONS

Other methods will have to be sought to induce complete immunity.

PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD-DR&E(AK)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DMB'S INSTR'N	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62172A	3M162172A810	00	002			
<del>XXXXXXXXXX</del>	62772A	3M762772A810	00	002			
<del>XXXXXXXXXX</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code)* (U) Prevention of Skin Disease Caused by Environmental Assaults on Skin							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 003500 - Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:		EXPIRATION:		PRECEDING		B. FUNDS (in thousands)	
A. NUMBER*				FISCAL		77	
C. TYPE: Not Applicable		4. AMOUNT:		YEAR		3.0	
A. KIND OF AWARD:		F. CUM. AMT.		CURRENT		64	
				78		3.0	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME* Letterman Army Institute of Research				NAME* Letterman Army Institute of Research			
ADDRESS* Presidio of San Francisco, CA 94129				ADDRESS* Department of Dermatology Research Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME* Akers, William A., COL, MC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5455			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Prystowsky, Stephen D., MAJ, MC			
				NAME: Grekin, David A., MAJ, MC POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Ultraviolet light; (U) Sweat; (U) Contact Dermatitis; (U) Skin; (U) Friction; (U) Blisters; (U) Water Immersion; (U) Miliaria; (U) Occlusion							
23. TECHNICAL OBJECTIVE* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) (U) The objectives are to develop prophylactic measures against the assaults of heat, humidity, friction, pressure, occlusion, water immersion, sweat, and ultraviolet light alone and in combination on soldiers' skin. Diseases like friction blisters, paddy foot, prickly heat rash, athlete's foot, jock itch, skin infections, and sunburn result in epidemics among soldiers and cause high morbidity, man-days lost, and interfere with performance of mission.							
24. (U) The natural history of the diseases will be studied. Promising prophylactic and therapeutic measures will be tested in the laboratory and the field.							
25. (U) 76 10 - 77 09. Paddy foot was successfully produced in 3 of 3 volunteers in 74 hours of continuous immersion in the Sacramento River Delta sloughs, but no paddy foot was induced in 2 volunteers by 80 hours who immersed their feet in distilled water while housed in an environmental chamber reproducing the field water temperature conditions. A survey was conducted at Ft. Leonard Wood, MO. (479 recruits, 151 recruit outpatients in the troop podiatry clinic, and 301 hospital charts). Cross-prevalence surveys revealed that 59 percent of recruits had at least 1 blister (43 had multiple blisters), 400/1000/year visited the troop podiatry clinic, while 40/1000/year were hospitalized for complications of friction blisters requiring 3.3 days of hospitalization plus 5 days of no physical training. Blisters of the posterior and lateral heel and the anterior ankle became infected most frequently. We estimate that blisters in recruits cost the armed services 1.42 million dollars per year.							

# ABSTRACT

PROJECT NO. 3M762772A810 Military Skin Disease  
WORK UNIT NO. 002 Prevention of Skin Disease Caused by  
Environmental Assaults of Skin

The following investigations have been conducted under this work unit:

STUDY NO. 1 Epidemiology of friction blisters in soldiers

STUDY NO. 4 Water immersion effects of prolonged water exposure  
on soldiers skin

STUDY NO. 1 The natural history of epidemiology of friction blisters in recruits has been determined. This investigation was recently completed at Fort Leonard Wood, Lackland Air Force Base, and the Marine and Navy Recruit Training Center, San Diego. Studies were performed on the field during different phases of basic combat training (BCT). Data were also collected at podiatry clinics and hospitals. Friction blisters on the posterior, medial and lateral heel and the anterior ankle cause the most morbidity and are more likely to become infected. A study designed to prevent friction blisters by applying glutaraldehyde to these areas will begin in March 1978.

STUDY NO. 4 Warm-climate water immersion injuries produce high rates of disability in soldiers exposed for prolonged periods to wet terrain. An experimental model has been developed for the injury to the sole of the foot. A more serious form of injury (paddy foot) involves the top of the foot and the lower leg. Little is known concerning the pathogenesis of this type of injury. Both injuries were produced in three volunteers from our department on a 74-hour field study in the Sacramento River Delta. However, in a recent study conducted in our laboratory, we failed to produce the more serious injury in 2 volunteers who immersed one foot in water continuously for 80 hours. This casts doubt on early hypothesis theories that water immersion injuries are due solely to water toxicity.

## BODY OF REPORT

WORK UNIT NO. 002

Prevention of Skin Disease Caused  
by Environmental Assaults on Skin

STUDY NO. 1

Epidemiology of friction blisters  
in soldiers

### PROBLEM

Friction blisters cause high rates of disability in the military, especially among recruits in training and in seasoned troops suddenly mobilized to stressful environments of sustained operations. In 1901, Munson reviewed the impact of friction blisters on military operations and stated that blisters may rapidly render large numbers of soldiers unfit for service and diminish the effective force at the beginning of the campaign. As many as 25-30% of troops may sustain injury to the feet during the first few days of forced marching. He noted that at one time 30,000 German soldiers were unfit to perform field service due to friction blisters. Development of a prophylaxis for friction blisters has a high priority in the recently published STOG-78 in that blisters must be prevented early (DO-15) to permit greater strategic mobility and improve troop performance in the stressful environment of sustained operations.

### RESULTS AND DISCUSSION OF RESULTS

Cross prevalence studies performed in the field at Fort Leonard Wood revealed that 60% of 479 soldiers examined during basic combat training (BCT) had at least one blister. Thirty-three percent had 2 or more blisters. The location distribution was: heel (34%), ankle (10%), dorsum of foot (4%), toes (51%), sole (13%). New blisters most commonly developed during the third and fourth week of training when the recruits were forced marched to the rifle range. Only 8% of soldiers with blisters in the field reported to podiatry clinic. There are several explanations for this. (1) Toe and sole blisters, though common, rarely cause significant problems; and (2) most recruits will tolerate moderate pain and even infection rather than go to foot sick call, since they fear being recycled and leaving their platoon to which they strongly identify.

We examined 151 soldiers who reported to the podiatry clinic for treatment of friction blisters. The location distribution of the blisters in the podiatry clinic study was on the heel (76%), ankle (36%), dorsum of foot (14%), toes (44%), and sole (32%). Sixty-six percent had 2 or more blisters. Patients with posterior, medial and lateral heel, and anterior ankle blisters (greater than 1 cm in size) most frequently presented to the podiatry clinic. Pain, as well as possible infection (39%), was a common chief complaint. The rate of podiatry clinic visits per year for blister debridement



at Fort Leonard Wood was calculated at 400/1000/year. Ten percent of recruits seen at the podiatry clinic for friction blisters were hospitalized for friction-blister-related cellulitis.

During an 18-month period, we examined the hospital records of 301 recruits admitted with a diagnosis of foot cellulitis. At least 90% of such patients were directly related to friction blisters. (Comprehensive prospective hospital studies are nearly completed at Fort Leonard Wood, Lackland Air Force Base, and the Marine and Navy Recruit Training Center, San Diego). The median hospital stay was 3.3 days (range of 1 to 26 days) followed by restricted activity for an additional 4 days. Eighty-five percent of hospitalized patients had either heel or ankle blisters. Sixty-seven percent of hospitalizations occurred prior to the fourth week of training.

In addition to the morbidity caused by friction blisters in BCT, there is considerable cost to the Department of Defense. At Fort Leonard Wood in 1976, the cost of hospitalization was \$82 per recruit/day; the cost of outpatient visits was \$13/day; and the cost of BCT per soldier was \$38/day. Calculations made from these data (based on the average strength figure of 6,000 soldiers in BCT at any one time at Fort Leonard Wood in 1976), revealed that \$142,000 per year were spent on friction blisters and related cellulitis. Based on strength figures for the Army, Navy, Air Force, and Marines, the estimated cost, in 1976, to the Department of Defense for friction blisters and related cellulitis was between 1.5 and 2 million dollars. We have unpublished data from our field surveys that show the friction blister problem is similar in the Army, Navy, Marines and Air Force.

#### CONCLUSIONS

Friction blisters and friction blister related cellulitis are a well documented major military medical problem. These data have made it possible to design prospective prophylactic studies. Glutaraldehyde and other such compounds which may reduce the incidence of friction blisters in soldiers are used as prophylactic agents.

#### RECOMMENDATIONS

Study No. 2 entitled, "Prevention of Friction Blisters in Soldiers with Glutaraldehyde" has been submitted to the Human Use Committee, TSGO. Pending its approval, the pilot study will begin in March 1978.

#### PUBLICATIONS

1. MAIBACH, H.I., and S.D. PRYSTOWSKY. Glutaraldehyde (pentanedial) allergic contact dermatitis: Usage test on sole and antecubital fossa-regional variations in response. Arch Dermatol 113: 170-171, 1977.

PROBLEM

Injuries to the foot known as "warm water immersion foot" and "tropical immersion foot" (paddy foot) accounted for up to 50% of the total man-hours lost among troops operating in the flooded terrain of the Mekong Delta in South Vietnam. Similar statistics were reported in United States troops fighting to retake the Philippines. Additionally, a potential problem exists in the Arctic region and other cold climates where combat troops may be forced to wear insulated boots for prolonged periods. Waterproof footwear retains sweat so that, in effect, the foot is continuously immersed in warm water. No method is known for preventing these injuries short of reducing the duration of exposure to water. This has the serious disadvantage of being a major limiting factor in conducting ground combat operations in wet terrain. A convenient easily controlled method of experimentally inducing warm water immersion foot of the soles in volunteers has recently been developed and may prove useful in future study of this injury. However, no model exists for the more severe, acute form of injury, tropical immersion foot, although it is possible to produce this entity experimentally in the field.

RESULTS AND DISCUSSION OF RESULTS

A pilot study was performed in the swampy terrain of the Sacramento River Delta in an area where the Navy and Marines conduct riverine training. Three volunteers spent 74 h walking in water and mud and sleeping with their feet in water. The acute form of water immersion injury (paddy foot) was produced in all three volunteers. In a second pilot study two volunteers each immersed one foot in water continuously for 80 hours in the laboratory environmental room. Water temperature was controlled by a circulating water bath being the same temperatures experienced in the earlier field study. While both volunteers developed the mild form of injury involving the sole of the foot, neither developed any sign of the more severe injury. The results of this experiment cast considerable doubt on early theories that the pathogenesis of these injuries is due entirely to water toxicity.

CONCLUSIONS

Warm water immersion foot and tropical immersion foot can occur in California as well as the tropics. The ease with which these injuries can be produced in soldiers operating in swampy terrain further emphasizes their importance to the Army. Failure to produce tropical immersion foot in volunteers in a laboratory setting suggests more complex factors than simple water toxicity in the etiology of this entity.

### RECOMMENDATIONS

Work should continue on the effects of prolonged water immersion on human skin because of its military importance. Factors other than simple water toxicity should be investigated, especially the role of temperature and any relationship between tropical immersion foot and classical trench foot; chemicals in the water; bacteria and fungi; the role of the boot; and the friction and pounding produced by marching in sucking mud.

### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OB 6800	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISB'S INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62172A	3M162172A810		00		003	
<del>XXXXXXXXXX</del>	62772A	3M762772A810		00		003	
<del>XXXXXXXXXX</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) More Effective Topical Repellents Against Malaria - Bearing Mosquitoes							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 - Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
67 11		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
d. NUMBER *				FISCAL YEAR		33	
c. TYPE: Not Applicable				CURRENT		38	
e. KIND OF AWARD:				78		2.4	
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
ADDRESS: Presidio of San Francisco, CA 94129				ADDRESS: Cutaneous Protection Division			
				Department of Dermatology Research			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME: Reifenrath, William G., CPT, MSC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5485			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Akers, William A., COL, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Tropical Diseases; (U) Topical Repellents; (U) Human Volunteers; (U) Insect Repellent; (U) Mosquito; (U) Skin; (U) Stratum Corneum; (U) Polymer Formulations							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objectives are to discover a long-lasting water and abrasion resistant, topical repellent formulation that will protect soldiers against malaria-bearing mosquitoes and other vectors of militarily important diseases; to develop in vitro test methods to determine physical properties of repellent formulations which are tested in the in vivo screening program; and by correlating the in vitro and in vivo test results, to predict evaporation, penetration, and repellent-skin interactions which will aid in designing new repellents.							
24. (U) Repellent evaluation is coordinated with the Department of Tropical Medicine. Physical properties and efficacy of repellents and formulations will be determined by partition coefficient, relative solubility, volatility, surface activity, and duration on animals and man. With the use of a computer base, for statistical analyses of repellent test results, individual characteristics which can be enhanced to promote longer duration of repellent protection will be determined.							
25. (U) 76 10 - 77 09. A formulation laboratory is being established and new in vitro tests based on thin layer and gas chromatography for solubility and partition coefficients are being developed to evaluate new repellent-vehicle combinations. Minimum effective dose and dry protection time determinations done in the hairless dog for four repellents yielded data comparable to analogous test results in human volunteers. A field study was conducted to compare three experimental repellents versus deet against Anopheles mosquitoes. In decreasing order of protection were carbamine, SRI-6, sulfonamide, deet.							

<sup>a</sup>Available to contractors upon originator's approval.

# ABSTRACT

PROJECT NO. 3M762772A810 Military Skin Diseases  
WORK UNIT NO. 003 More Effective Topical Repellents  
Against Malaria-Bearing Mosquitoes

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Mosquito repellent data management system
- STUDY NO. 2 The hairless dog as an animal model for repellent studies
- STUDY NO. 3 Field testing of repellents against Anopheles mosquitoes.

A large amount of repellent test data has previously been stored in a remote file management system. Study No. 1 represents our efforts to store these data in the Statistical Package for Social Science data management system. This system allows sophisticated statistical analysis to identify important human factors in repellent efficacy. Under Study No. 2 we examined the Mexican hairless dog as an animal model for repellent studies. The minimum effective dose and dry protection time for a number of repellents was determined. The dogs ranked repellents in similar order as comparable tests with volunteers. In Study No. 3, three experimental repellents were found superior to deet in a small field trial against Anophelene mosquitoes.

## BODY OF REPORT

WORK UNIT NO. 003

More Effective Topical Repellents  
Against Malaria-Bearing Mosquitoes

STUDY NO. 1

Mosquito repellent data management  
system

### PROBLEM

A large amount of data relating to the physical properties of repellents and effectiveness against mosquitoes has been collected over the past seven years. These data have been loaded into a Remote File Management System (RFMS) as described in the 1976 Annual Report. Although this computer management system can provide effective retrieval, the system has limited statistical analysis options. These statistical analyses are needed to identify exploitable factors for design of better mosquito repellents to be used by military personnel in training or combat in infested areas.

### RESULTS AND DISCUSSION OF RESULTS

In FY 76, repellent, volunteer, and test data covering 6 years of mosquito repellent research were coded into a computer storage system known as the Remote File Management System (RFMS). Routine retrievals from this system (individual profiles, means, standard deviations) and data additions have continued to the present. Although this system provides an excellent management system for a historical file of the data, its statistical capability is limited. Therefore variables in the RFMS have been recoded, linearized, and data are being loaded into the Statistical Package for Social Science (SPSS) system. This system will not only store and allow retrieval of repellent data but will also provide more sophisticated statistical analysis of the data (i.e. multiple regression analysis). The value of the SPSS system has been extended by new variables which have been added to the system by the investigators in the Departments of Dermatology Research and Tropical Medicine.

### CONCLUSIONS

The SPSS will not only provide a historical file for mosquito repellent data, but will also provide sophisticated statistical analysis.

### RECOMMENDATIONS

Off-loading of data from the RFMS system to the SPSS system should continue. Additional data obtained in future repellent tests should be added to the SPSS management system. After the SPSS loading is completed, multiple regression analysis of repellent data should be done to identify exploitable factors for the design of better mosquito repellents.



## PUBLICATIONS

None

STUDY No. 2

The hairless dog as an animal model  
for repellent studies.

## PROBLEM

In our search for a more effective mosquito repellent, we found it necessary to develop an animal model which could be used to screen the duration of new repellents before toxicological studies are done. The Mexican hairless dog was investigated for this purpose. Minimum effective dose and repellent dry protection times were determined in both the dog and volunteers in analogous tests for various repellents to assess the validity of the model.

## RESULTS AND DISCUSSION OF RESULTS

A dry protection test analogous to the standard 4-site human test for duration of repellents was conducted on Mexican hairless dogs. The repellents p-deet, m-deet, SRI-6, carbamide, sulfonamide, 6-12, and the Stanford Research Compounds 835-9B, 7048-105, 835-17A, 835-19C, 835-23A were tested.

Two 7 x 7 cm<sup>2</sup> sites were drawn on both sides of the dog's back and known weights of the repellents were applied with a 1 cc syringe and spread with a clean glass rod. Testing began one hour after application. An adhesive-backed foam pad was affixed around the perimeter of 2 trap door openings of a twin compartment cage (each compartment is 7.5 x 7.5 x 15 cm and contains 50 female *Aedes aegypti* mosquitoes). Two of these twin compartment cages are placed over the 4 sites with the foam pad touching the perimeter of the repellent treated sites. The trap doors are opened simultaneously for a 3-min time interval. If one of the sites receives 2 bites in one test period or 2 bites in consecutive test periods, the site has failed and the time is recorded as the dry protection time. The results of the Dry Protection Test for duration in the dog are as follows (repellent, duration in hours, dose in mg/cm<sup>2</sup>): Indalone, 0.9, 0.32; carbamide 1.0, 0.32; dimethylphthalate, 2.6, 0.32; 2-ethylhexanediol, 3.3, 0.32; SRI-6, 4.7, 0.32; p-deet, 5.2, 0.32; carbamide 5.5, 0.96; m-deet 7.2, 0.32, sulfonamide, 8.3, 0.32; 835-9B, 0.7, 0.32; 7048-105, 1.0, 0.32; 835-17A, 0.9, 0.32; 835-19C, 2.4, 0.32; 835-23A, 0.6, 0.32; 165-67, 5.0, 0.32. Comparable results for the standard repellents were seen in volunteers as follows (repellent, duration in hours, dose in mg/cm<sup>2</sup>): Carbamide, 2.0, 0.32; 2-ethylhexanediol, 3.9, 0.32; SRI-6, 2.4, 0.32; p-deet, 5.0, 0.32; m-deet, 5.8, 0.32; sulfonamide, 6.9, 0.32. (With sulfonamide 40% of the applications to dogs lasted longer than 10-13 hours; 25% of the applications of sulfonamide to volunteers lasted longer than 12 h). In our Dry

Protection Time testing of the Mexican hairless dogs, some repellents had a much longer duration than others. Many factors affect this duration but one that we found was most important was the minimum effective dose (MED). The MED is defined as the minimum dose of repellent required to repel mosquitoes. A repellent solution is applied at 0.10 mg/cm<sup>2</sup> and is allowed to dry for 15 minutes. The site is then exposed to a mosquito test cage (7.5 x 7.5 x 15.0 cm) containing 50 female *Aedes aegypti* mosquitoes for 3 min. If the site receives 2 bites, a 0.15 mg/cm<sup>2</sup> concentration of the same repellent will be applied to another test site, allowed to dry and tested again. Depending on the success or failure of this test a lower or higher (respectively) concentration for the repellent will be tested in order to bracket the MED. If the initial application does not receive 2 bites, a 0.05 mg/cm<sup>2</sup> repellent concentration will be applied and tested. Again, depending on the success or failure of this test the last 2 sites will bracket the MED. In the dog, the lower the MED or the lower the concentration of repellent required to repel mosquitoes, the longer the duration (DPT). The following data illustrate this (repellent, MED, DPT): sulfonamide, 0.024, 8.3; m-deet, 0.07, 7.2; SRI-6, 0.12, 4.7; 7048-105, 0.18, 1.0; 835-17A, 0.41, 0.94; 835-9B, 0.54, 0.69; carbamide, 0.61, 1.00.

#### CONCLUSIONS

The Mexican hairless dog shows promise as a model for repellent studies in the laboratory. In comparable tests, the dogs ranked repellents in the same order as did volunteers.

#### RECOMMENDATIONS

Work should continue with the Mexican hairless dog to verify its usefulness as an animal model for repellent studies. Other species of mosquitoes should be investigated and the results compared with tests in volunteers. Percutaneous penetration studies with labeled compounds should be done in the dog and the results compared with published results in man and other species.

STUDY NO. 3

Field testing of repellents against  
*Anopheles* mosquitoes

#### PROBLEM

The presently available mosquito repellent now in use by the military (deet) is excellent except for the fact that it washes off and sweats off rapidly in hot humid environments. The Cutaneous Protection Division, LAIR, is trying to improve repellents by increasing the length of time one application will protect a man against biting by malaria-bearing mosquitoes and other disease carrying insects.

## RESULTS AND DISCUSSION OF RESULTS

The test method used was the LAIR four-site field technique, which involves the area from the wrist to the elbow of each forearm divided into equal sections separated by an adhesive back foam strip to prevent mixing of repellents via diffusion across the skin.

Four different repellents, including one control (m-deet), were applied in known weight per unit area to 4 sites on each of 4 individuals at time intervals ranging from 4 h to 16 h before the evening test period. Tests were conducted over a 4-day period. The test subjects underwent mild exercise during the day. The individuals were then exposed to a natural population of Anopheles mosquitoes during the early evening hours. The time was recorded each time an individual received a bite. When 5 bites were received on a particular site, the repellent on that site was defined to have failed.

This test was conducted in an area bordering rice fields near Colusa, California, from 29 August - 1 September 1977. The results of this test expressed as average hours of protection for each repellent are as follows: carbamide, 13.5; SRI-6, 11; sulfonamide, 9. The control repellent, m-deet, failed to protect 3 of the 4 individuals during the 4 days of testing even at pretreatment intervals as short as 4 h.

The species and biting rate of mosquitoes collected from untreated human arms at Colusa, California, during the 4 days of testing between 1915 - 2045 h are as follows: Anopheles freeborni, 78%, 88 bites/h, Aedes dorsalis, 4%, 5 bites/h; Aedes vexans, 15%, 17 bites/h; Culex tarsalis, 3%, 4 bites/h. Biting rates are based on the average of 13 (5 min.) biting collections.

## CONCLUSIONS

In this field trial, carbamide provided superior protection against Anopheles mosquitoes. The 2 other test repellents, SRI-6 and sulfonamide, also provided better protection than deet.

## RECOMMENDATIONS

Field trials such as this should be done routinely, since this test provides the closest approximation to actual repellent use conditions. Since many tests are being examined in the laboratory to screen repellents, field tests provide valuable input to insure their validity during repellent development. Large scale field tests with these promising new repellents should be done.



#### PUBLICATIONS

1. SPENCER, T.S., K.L. ZELLER, C.F. BRODEL, and W.A. AKERS.  
Analysis of four-site method for testing mosquito repellents.  
In: Proceedings and Papers of the Forty-fifth California Mosquito  
Control Association Inc., Palm Springs, CA., 13-16 February, 1977  
(in press).
2. SPENCER, T.S., J.A. HILL, W.A. AKERS, and G. BJORKLAND.  
Studies of repellent formulations with n, n-diethyl-m-  
toluamide. In: Proceedings and Papers of the Forty-fifth California  
Mosquito Control Association Inc., Palm Springs, CA., 13-16  
February 1977 (in press).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OB 6912	77 10 01	DD-DR&E(AR)1636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'N INST'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO. CODES <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		62172A		3M162172A810		00	
XXXXXXXXXX		62772A		3M762272A810		00	
XXXXXXXXXX		CARDS 114 f				004	
12. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Biochemical Studies on Prevention and Control of Skin Disease in Military Personnel							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 - Clinical Medicine							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
68 07		CONT		DA		C. In-House	
18. CONTRACT GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				B. PRECEDING		C. FUNDS (In thousands)	
B. NUMBER <sup>a</sup>				FISCAL YEAR		77	
C. TYPE Not Applicable				CURRENT		3.0	
D. KIND OF AWARD				78		3.0	
E. CUM. AMT.						79	
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
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ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Department of Dermatology			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: POC: DA			
				NAME:			
24. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup> (U) Skin Disease; (U) Fungal Infection; (U) Water Immersion; (U) Protective Agents; (U) Biochemistry; (U) Occlusion; (U) Insect Repellent; (U) Miliaria							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Since 1942, the Army documents a rate of skin disease between 15 and 70% in military forces. Even today during combat operations the skin is exposed to adverse environmental conditions (poor sanitation, heat, occlusion), which may profoundly alter biological characteristics increasing the susceptibility of skin to damage from infection or trauma within 2 to 10 days. It is the purpose of this work unit to study biochemical and biophysical skin parameters under normal conditions, to study alterations of these parameters due to adverse environmental conditions, and to develop preventive measures to reduce skin disease in military populations.</p> <p>24. (U) The project will focus on surface aspects of skin diseases. Efforts will be made (1) to develop or improve biochemical and biophysical methods, (2) to apply the methods to the study of bacterial and/or fungal infection and to causes of casualties such as trench foot or miliaria (which by themselves may not be incapacitating but may be predisposed to infections and other skin diseases), and (3) to apply the evolved techniques to influence the retention and/or efficacy of insect repellents.</p> <p>25. (U) 76 10 - 77 09. Studies continued on characterizing and quantitating inhibitors of fungal infection (<i>Trichophyton mentagrophytes</i>) in skin lipid extracts from feet. Glycerol levels were determined which suggest that glycerides constitute a small fraction in lipids from the feet unlike other skin areas. Two rapid methods (agar slide method and electronic cell counting of suspension cultures) have been developed to quantitate germination of spores and growth of hyphae of <i>T. mentagrophytes</i>. Phase microscopic photography has been standardized and computer programs developed. The two methods have been applied to quantitate the activity of lipophilic inhibitors on <i>T. mentagrophytes</i>.</p>							

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

# ABSTRACT

PROJECT NO. 3M762272A810 Military Skin Diseases  
WORK UNIT NO. 004 Biochemical Studies on Prevention  
and Control of Skin Disease in  
Military Personnel

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Biological activity of human skin lipids in relation to fungal disease
  - EX-1 Development of method
- STUDY NO. 2 Lipid sampling from the human skin surface
- STUDY NO. 3 Dermatological problems of the soldier during World War II as documented in confiscated German documents
- STUDY NO. 16 Triglyceride content of skin lipids in relation to fungal infection

STUDY NO. 1 Two screening methods were developed to study the growth and development processes of Trichophyton mentagrophytes in the presence or absence of lipophilic inhibitors. A new generalized data base management system was designed and tested to allow rapid evaluation of size distributions.

STUDY NO. 2 A protocol has been developed to study lipid sampling from the human skin surface.

STUDY NO. 3 Our present evaluations indicate that confiscated German war documents will give significant insight into dermatological problems of troops in the European and Mediterranean theaters during World War II. These evaluations may provide the necessary information to plan effective preventive strategies for potential conflicts in OCONUS.

STUDY NO. 16 The objectives have been to develop a specific method for quantitation of triglyceride levels and to apply it to skin lipid extracts from volunteers with and without fungal disease on their feet. There was no statistically significant difference in triglyceride levels observed between groups; triglyceride levels appear to be much lower than those observed on the forehead or back and as previously speculated.



## BODY OF REPORT

WORK UNIT NO. 004

Biochemical Studies on Prevention  
and Control of Skin Disease in Military  
Personnel

STUDY NO. 1

Biological activity of human skin  
lipids in relation to fungal disease

EX-1

Development of method

### PROBLEM

Fungal infection on feet and legs reached epidemic levels in soldiers during the Vietnam conflict and during World War II. It appears that outbreaks of the disease correlated with environmental factors. In Vietnam the epidemic could be controlled through use of therapeutic doses of griseofulvin. However, according to the older literature this fungistatic inhibitor is a potential carcinogen and griseofulvin thus may no longer be available to troops. Previously, however, studies from this department have shown that volunteers exposed to the same environment can be grouped into three populations: (a) those that have never had fungal disease, (b) those that had fungal disease occasionally, and (c) those that are afflicted chronically by the fungus. The controlling factors for fungal disease are thus largely unknown, although it has often been postulated that inhibitors or promoters found in skin lipids play an important role in the control of fungal disease.

The inhibitory effect of certain pure fatty acids against fungi grown in in vitro systems has been known for some time. However, major technical difficulties so far have precluded effective testing of large numbers of lipophilic inhibitors of fungal growth.

The control of epidemic fungal disease in troops and the design and testing of new, preventive measures thus heavily depends on the development of new and simple methods which can be used to test these inhibitors.

### RESULTS AND DISCUSSION OF RESULTS

Two new systems - an agar slide method and a shake culture method - have been developed to study in vitro growth of Trichophyton mentagrophytes in the presence of lipophilic inhibitors.

Agar slide method. In two experiments a measured number of T. mentagrophytes spores were applied onto 1 mm thick agar slabs supported by microscopic slides which were housed in a sterile petri dish assembly. These were incubated at 30 C in a moist chamber. Germination of spores was observed with a Wild inverted phase microscope. Photographs were

taken with a Zeiss camera, electronic flash assembly and appropriate density filters. Growth of fungus was followed over a period of 7 to 10 days. Photographic procedures have been standardized. Films from experiments, in which 4 different agar media and 6 different lipophylic model growth substrates and inhibitors were used, are being evaluated.

Shake culture method. A Coulter Counter model ZB equipped with channelizer, interface and teletype unit was used to measure culture of T. mentagrophytes. Spore counts and size distributions were made and recorded on paper tape. Computer programs were written to convert spore counts in individual windows (volume classes) to relative concentration and cumulative frequency. Programs were also written to have the computer plot the window number and/or logarithm of the window number versus the cumulative frequency. Programs were written to have the computer do a probit-analysis of the data. All subsystems are operative and the new method yields rapid and highly reproducible results. Measurements in which the Coulter Counter Amplification control was varied allowed for the evaluation of particle or mycelia contamination in spore preparations. In conjunction with filtration and low speed centrifugation a procedure has been developed to produce faster, better controlled, and more uniform spore preparations. Using these Coulter Counter techniques, we have performed a number of time studies in which T. mentagrophytes was cultivated in two different media in the presence or absence of a lipophylic inhibitors.

#### CONCLUSIONS

Preliminary evaluation of photographic negatives indicates that morphological and photogrammetric evaluation of the agar slides will be a powerful new tool to obtain detailed information on growth inhibitors of T. mentagrophytes. Computer aided evaluations of changes in size distributions of fungal growth is also a new and powerful tool for rapid evaluation of the efficacy of growth inhibitors.

#### RECOMMENDATIONS

A few additional verifications of the new agar slide and the Coulter Counter methods should be made. Thereafter, the two methods should be used to evaluate the efficacy of new antifungal agents and the inhibitory effect of skin-lipid extracts from volunteers with and without fungal disease of their feet.

#### PUBLICATIONS

None

STUDY NO. 2

Lipid sampling from the human skin surface

#### PROBLEM

Ways to prevent or minimize the damage that agents of the natural environment inflict on the soldiers skin require knowledge of the biochemical composition of the skin surface and its biophysical behavior. Understanding the biochemical composition requires collection of biomolecules such as lipids from the skin surface. Lipid samples are obtained from human volunteers under this work unit or in conjunction with other work units in the department. Experiments on the collected samples are done to test predetermined or retrospectively formulated hypotheses.

#### RESULTS AND DISCUSSION OF RESULTS

Sampling procedures have been standardized and the necessary protocol to collect lipid samples from the skin surface of human volunteers has been developed.

#### CONCLUSIONS

This is a new study protocol which awaits approval by The Surgeon General.

#### RECOMMENDATIONS

The procedures should be used to collect lipids in relation to the development of protective strategies against abrasion, friction blisters, warm water immersion foot, and its variants and infectious diseases of the skin of soldiers.

#### PUBLICATIONS

None

STUDY NO. 3

Dermatological problems of the soldier during World War II as documented in confiscated German documents

#### PROBLEM

American records of militarily relevant dermatological problems during World War II are rather sketchy. This is especially true for records with regard to prevention of endemic disease in relation to geographical areas and prevention of occupational dermatitis. In contrast, upon surrender of German troops, extensive data on medical problems and in particular dermatological problems of military forces were recovered by U.S. intelligence. Microfilms of these captured



German documents are housed in the Armed Forces Medical library in Washington, DC, but have never been analyzed.

It is the purpose of this study (a) to analyze German Army documentation from World War II with regard to relevance of potential dermatological problems of the soldier and (b) to provide the basis for planning dermatological counter measures in order to maintain a healthy force structure in central Europe.

#### RESULTS AND DISCUSSION OF RESULTS

Preliminary screening of confiscated German documents (OKH-20 documents) has been accomplished. German notes to the documents suggest that nine microfilms contain potentially relevant information for the Department of Dermatology Research. The microfilms are on order from the National Archives and Records Service, Washington, DC.

#### CONCLUSIONS

Preliminary analysis of German military documents has shown clearly that much information will be gained about hazards which the civilian dermatologist or the military dermatologist in times of peace may not likely see.

#### RECOMMENDATIONS

In Phase I microfilms should be read and relevant information on military dermatological problems should be recorded, including leads to other relevant sources. If warranted, an information storage system should be developed to allow development of a data bank. Documents identified in Phase I should be abstracted in German that will be translated into English. Where necessary and/or pertinent, more extensive translation of documents should be performed in order to define potential future dermatological problems and in order to design preventive strategies effective against adverse environmental effects in Central Europe and the Mediterranean.

#### PUBLICATIONS

None

STUDY NO. 16

Triglyceride content of skin lipids  
in relation to fungal infection

#### PROBLEM

Triglycerides on the skin surface are constantly exposed to esterases derived from skin, from bacteria, and possibly fungi. Triglycerides are thus one of the precursors of free fatty acids, some of which are found inhibitory to Trichophyton mentagrophytes. Triglycerides are

synthesized by sebaceous glands or are structural components of the membrane systems of epidermal cells. In areas of the face or back triglyceride levels have been determined by nonspecific methods and range between 20 and 50% of the total lipid present. However, no triglyceride data are available for the leg or the foot even though the incidence of fungal disease in these areas of the human body was high in Vietnam and Europe.

In this study the hypothesis is tested that volunteers with chronic fungal infection on their feet do not have a higher concentration of triglycerides than volunteers who never had an infection. The present study provides a quantitative evaluation of this hypothesis.

#### RESULTS AND DISCUSSION OF RESULTS

Triglycerides were determined in skin lipid extracts based on our modification of the method of Bucolo and David (Clin Chem 19: 476, 1973). Enzymatic hydrolysis of the triglyceride to glycerol and free fatty acids was not successful since a series of emulsifying agents at optimal concentration inhibited the lipases. Alkaline hydrolysis in toluene-ethanol solution was, however, successful. The glycerol was phosphorylated, with glycerokinase and the resulting ADP reacted with phosphoenolpyruvate to yield pyruvate which was reacted with reduced nicotinamide-adeninedinucleotide in the presence of lactate dehydrogenase. The change in absorbance at 340 nm was recorded with a Cary 15 recording spectrophotometer and absorbance change was found to be proportional to the concentration of glycerol. Computer programs for efficient calculation of glycerol concentrations, tabulation, statistical evaluation, and ranking of samples, were adapted from previous studies. Percent triglyceride in our foot lipid extracts is much lower than in skin extracts from other parts of the body. The triglyceride level for the sole portion is somewhat higher than that for the toe portion of the foot, but the difference is statistically not significant. This difference was found in all extracts of the three groups of volunteers, i.e. those who never had a fungal infection (V), those who had no active lesion (E) and those who had a chronic infection (C). Our data indicate that there is no statistically significant difference in triglyceride content per area between groups V and E, groups V and C, and groups E and C.

#### CONCLUSIONS

Our measurements are the first data where a specific method was used to determine the content of triglycerides on a human surface. Since no artifacts of the method could be detected it is of significance that the triglyceride level on the plantar and dorsal surface of the foot is much lower than previously reported values from the forehead or back. From the results it appears that fungal infection on the foot has no influence on the triglyceride levels of surface lipids.

### RECOMMENDATIONS

Triglyceride determinations with the enzymatic method should be verified for the foot and measurements should be extended to the back and to other areas of the body surface where fungal and bacterial disease is prevalent.

### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMMARY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3M162172A810	00	006			
<del>XXXXXXXXXXXX</del>	62772A	3M762772A810	00	006			
<del>XXXXXXXXXXXX</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Skin Diseases Among Soldiers							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 - Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
68 01		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE			
a. DATES/EFFECTIVE:				PRECEDING			
b. NUMBER <sup>a</sup>				FISCAL			
c. TYPE: Not Applicable				77			
d. KIND OF AWARD:				CURRENCY			
e. CUM. AMT.				78			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				Cutaneous Protection Division			
RESPONSIBLE INDIVIDUAL				Department of Dermatology Research			
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TELEPHONE: (415) 561-3600				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
21. GENERAL USE				NAME <sup>a</sup> Akers, William A., COL, MC			
Foreign Intelligence Not Applicable				TELEPHONE: (415) 561-5455			
				SOCIAL SECURITY ACCOUNT NUMBER			
				ASSOCIATE INVESTIGATORS			
				NAME: Prystowsky, Stephen D., MAJ, MC			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Human volunteers; (U) Occupation; (U) Diagnosis;							
(U) Skin; (U) Survey; (U) Soldiers; (U) Morbidity							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objectives are to determine the type and frequency of potentially disabling skin diseases among soldiers in various environments, to conduct trials of potential preventive and therapeutic agents against the common, disabling dermatoses that afflict military personnel, and to develop or improve methods of studying militarily relevant skin diseases under field conditions.							
24. (U) The proportion of patients with various diagnoses is being tabulated by using dermatology clinic visit data from four Army medical centers. Epidemiologic surveys of skin diseases in troop populations are conducted to determine the effect of environment, clothing, and other variables on the frequency and severity of lesions. New diagnostic techniques developed in the laboratory are tested in the field to determine their performance characteristics.							
25. (U) 76 10 - 77 09. A large collaborative prospective study of 1,158 people was performed to establish the exposure and contact sensitivity to nickel, neomycin, ethylenediamine, and benzocaine in a general population since soldiers frequently are exposed to these chemicals. Relationships between history of exposure and reactivity to a standard patch test are being analyzed. Data from a 3-year dermatological outpatient survey involving 146,395 initial visits and 51,155 followup visits are being analyzed. The most common diagnostic categories for active duty personnel are acne vulgaris, warts, pseudofolliculitis barbae, seborrheic dermatitis, allergic contact dermatitis, moles, dry skin, tinea pedis, dermatitis, and epidermal cysts.							

# ABSTRACT

PROJECT NO. 3M762772A810 Military Skin Disease

WORK UNIT NO. 006 Skin Diseases Among Soldiers

The following investigations have been conducted under this work unit:

STUDY NO. 1 Skin diseases among soldiers

STUDY NO. 10 Contact sensitivity to nickel, neomycin, ethylenediamine, and benzocaine in a general population: relationships between age, sex, history of exposure, and reactivity to standard patch test

STUDY NO. 1 A three-year survey of all outpatient visit diagnoses at four major Army dermatological training centers was completed and the data are being analyzed. There were 197,550 total visits including 146,395 initial visits. Active duty military personnel accounted for 22,847 initial visits (15.6 %). The 10 most frequent diagnoses for servicemen were acne vulgaris (13.8%), warts (8.5%), pseudofolliculitis of the beard (5.3%), dermatophilic fungal infections of the skin (4.8%), allergic contact dermatitis (4.1%), seborrheic dermatitis (3.9%), no diagnosis made at time of visit (2.9%), moles (2.7%), venereal diseases (2.7%), and dermatitis of unknown etiology (2.6%).

STUDY NO. 10 The prevalence of contact sensitivity to nickel, neomycin, ethylenediamine and benzocaine was determined in a general population. A pretested clinical questionnaire was administered in standard fashion to each volunteer before patch testing. Standard patch tests were administered to 1158 volunteers. The prevalence of positive patch tests was nickel (5.7%), neomycin (1.1%), ethylenediamine (0.43%), and benzocaine (0.17%). The low prevalence of neomycin sensitivity in the general population does not preclude its use for the treatment of pyoderma and miliaria in the military.

## BODY OF REPORT

WORK UNIT NO. 006

Skin Diseases Among Soldiers

STUDY NO. 1

Skin diseases among soldiers

### PROBLEM

Ninety-five percent of dermatological patients are outpatients. Dermatologists often do not have complete information on their outpatient workload, the amount of time spent caring for each skin disease, or an accurate measure of the morbidity produced by the skin diseases they treat. Records frequently cannot be retrieved. A computer supported dermatological outpatient data system provides additional information for the physician and administrator on the (1) outpatient patient load, (2) diagnostic and therapeutic problems encountered, (3) need for paramedical personnel and their training, (4) medical equipment and drug requirements, and (5) dispositions of patients. Such information is not available in the medical literature.

All outpatients attending the dermatology clinics at Brooke, Fitzsimons, Letterman, and Walter Reed Army Medical Centers completed a outpatient survey card for each visit entering dermographic information while the physician entered the diagnosis, initial or return visit, body area involved, special procedures, and an estimate of the time lost from duty because of the visit and the disease. Data were collected for FY 1973, 1974, and 1976. All cards were returned to LAIR where they were coded, key punched, and entered into the computer. The information was retrieved at monthly, quarterly, and yearly intervals. A printout consolidating the diagnoses and frequency distribution for all active duty personnel and all other people for initial and return visits for all three fiscal years has been compiled. Further analyses are being performed utilizing the Statistical Package for the Social Sciences (SPSS).

### RESULTS AND DISCUSSION OF RESULTS

The following information was received from the U.S. Army Health Information Systems and Biostatistical Agency, Ft. Sam Houston, TX, using the routine Army clinic visit statistical system. All clinic visits for the three years totaled 9,300,780 visits while inpatient and outpatient dermatology clinic visits totaled 244,978 or 3% of all clinic visits with 2,442,516 (26%) being active duty Army personnel.

Our dermatology outpatient survey showed 146,395 initial outpatient visits and a total of 197,550 visits including return visits. The unexpectedly low number of return visits was checked by two independent methods so that we know it is reasonably accurate. The discrepancy between our survey's number of visits and the routine



Army clinic visits statistics can be accounted for by their including inpatient visits, visits for prescription refills only, or patients not seeing a physician. Undoubtedly, some patients seen by a physician failed to have a card filled out in our survey but the number is unknown. Two spot checks showed that 97% of patients had cards filled out. Active duty personnel accounted for 22,847 initial visits (15.6%) and 30,723 total visits. Since these large teaching hospitals are referral centers, it is reasonable that the return visits should be lower than station hospital clinics.

Table 1 lists the most frequent 26 diagnoses (of 660 diagnoses recorded during the survey) for initial visits by active duty personnel from all military services. The "all patients" initial visits column included the active duty personnel visits. The relative rates observed reflect the peacetime Army in a temperate zone. Our earlier reports of our surveys from the 95 Evacuation Hospital, DaNang, Republic of Vietnam, and William Beaumont General Hospital, El Paso, TX, showed some variations due to climate, cultural environment, and occupation among soldiers (Table 2). Among the first 20 diagnoses there was agreement among 11 diagnoses in the three surveys; among the 40 most common diagnoses, agreement occurred in 23. Miliaria was never seen in El Paso while dry skin (asteatosis) never occurred in DaNang. The opportunity to acquire chancroid is higher in the Far East while syphilis lurks on our southern border. Acne, warts, ringworm infections, ingrown beard hairs, venereal diseases, and allergic contact dermatitis seem universally distributed. We are concerned about the LAIR survey finding that precancerous skin lesions occurred in 1.8% of military personnel and skin cancer occurred in 1.3% of active duty personnel.

The Army's experience in World War II and Vietnam indicated that dermatophytosis, pyoderma, miliaria, immersion foot syndromes, allergic contact dermatitis, insect infestations and bites, warts, and over-treatment dermatitis were common causes of large man-day losses. Common causes for hospitalization included pyoderma, infected eczematoid dermatitis, eczema, cystic acne, cellulitis, urticaria, and dermatophytosis.

Presently, the LAIR research program addresses problems involving dermatophytosis, pyoderma, allergic contact dermatitis, immersion foot syndromes, insect repellents, and friction blisters.

#### CONCLUSIONS

A 3-year, proportional rate survey of all dermatological outpatient visit diagnoses at 4 Army dermatological training centers has been completed and data are being tabulated and analyzed. Such new information is important for planning, teaching, and management purposes such as manpower, medical training, equipment, and drug requirements, identifying diagnostic and therapeutic problems, and patient disposition. The skin problems for the peacetime Army in the temperate zone were obtained but not for training posts. Over 30 dermatologists have



TABLE 1  
INITIAL VISIT DIAGNOSES

	Active Duty		All Patients	
	First	%	First	%
	N 22,847		N 146,395	
1. Acne vulgaris, all	3147	13.8	19831	13.5
2. Verruca vulgaris, warts, all	1943	8.5	11937	8.2
3. Pseudofolliculitis barbae	1203	5.3	1805	1.2
4. Dermatophytosis, fungal	1087	4.8	4537	3.1
5. Allergic contact derma- titis	927	4.1	5039	3.4
6. Seborrheic dermatitis	896	3.9	6158	4.2
7. No diagnosis made at this time	658	2.9	3589	2.5
8. Nevi (moles)	625	2.7	4988	3.4
9. Venereal diseases, all	610	2.7	2041	1.4
10. Dermatitis, unknown etiology	595	2.6	4370	3.0
11. Pyoderma, staph/strep	575	2.5	2665	1.8
12. Asteatosis, dry skin	447	2.0	3660	2.5
13. Herpes simplex, all	446	2.0	1573	1.1
14. Precancerous skin lesions	415	1.8	8839	6.0
15. Tinea versicolor	378	1.7	1283	0.9
16. Psoriasis	372	1.6	2670	1.8
17. Dyshidrosis, pomphylox	367	1.6	2024	1.4
18. Cyst, epidermal, sebaceous	326	1.4	2080	1.4
19. Atopic dermatitis	318	1.4	3216	2.2
20. Skin cancer, all	306	1.3	5234	3.6
21. Primary irritant contact dermatitis	305	1.3	1728	1.2
22. Lichen simplex chronicus	254	1.1	1776	1.2
23. Urticaria	240	1.0	1231	0.8
24. Seborrheic keratoses	236	1.0	6385	4.4
25. Scabies	231	1.0	584	0.4
26. Pityriasis rosea	225	1.0	1102	0.8
		<u>75.0</u>		<u>75.4</u>

TABLE 2

## Diseases Coded Active Duty Personnel

<u>El Paso, TX*</u>	<u>DeNang, RVN*</u>	<u>U.S. LAIR Survey</u>
1. Verruca	Verruca	Acne
2. Acne	Acne	Verruca
3. Dermatophytosis, all	Dermatophytosis, all	Pseudofolliculi- tis barbae
4. Pseudofolliculitis barbae	Pseudofolliculitis barbae	Dermatophytosis, all
5. Gonococcal urethritis	Penile ulcerative disease	Allergic contact dermatitis
6. Nevi	Miliaria	Seborrheic derma- titis
7. Urethritis, non-specific	Pyoderma, all types	No diagnosis
8. Contact dermatitis	Contact dermatitis	Nevi
9. Seborrheic dermatitis	Urticaria	Venereal diseases
10. Urticaria	Tinea versicolor	Dermatitis, un- known etiology
11. Dyshidrosis	Alopecia aerata	Pyoderma, all
12. Psoriasis	Psoriasis	Asteatosis
13. Cyst, epidermal	Atopic dermatitis	Herpes simplex, all
14. Tinea versicolor	Dyshidrosis	Precancerous skin lesions
15. Asteatosis	Candidiasis	Tinea versicolor
16. Pityriasis rosea	No diagnosis	Psoriasis
17. Syphilis, all types	Seborrheic derma- titis	Dyshidrosis
18. Dermatitis, unknown etiology	Herpes progentalis	Cyst, epidermal
19. Herpes progentalis	Molluscum contagiosa	Atopic dermatitis
20. Lichen simplex chronicus	Insect bites	Skin cancer
TOTAL 77.2%	84.0%	68.60%

\*Source: Jones, H.E., J.K. Anton, C.W. Lewis, et al. Environmental factors and skin diseases. Milit Med 141: 237-243, 1976.

been involved in collecting the data and many are now out supporting various posts. The proportional rate data will be most helpful for planning future short field surveys for incidence or prevalence of skin diseases among soldiers.

#### RECOMMENDATIONS

Further analysis of the data base as stored in the SPSS will involve tabulation of proportional skin disease for sex, race, age groups, military occupational specialties, military status, body area, and special procedures. Some diagnostic codes will be combined. The 3 years of data for each hospital will be tabulated separately. A technical report containing the tabulations, special studies, and interpretation is being prepared. Selected findings will be reported in the medical literature.

#### PUBLICATIONS

None

STUDY NO. 10

Contact sensitivity to nickel, neomycin, ethylenediamine, and benzocaine in a general population: relationships between age, sex, history of exposure, and reactivity to standard patch test

#### PROBLEM

A recent report on the epidemiology of contact dermatitis has been widely quoted and misinterpreted by many physicians and some persons in regulatory agencies. Some have assumed that a certain percent of the American adult population are allergic to nickel (11%), ethylenediamine (7%), neomycin (6%), and benzocaine (5%).

Soldiers may come in contact with all four of those common antigens. Neomycin probably reduces the infection rate of minor skin injuries and is widely used and accepted by military physicians. Impetigo and pyoderma are common military medical problems in which neomycin may be helpful. Pretreatment with neomycin appears to prevent the development of miliaria rubra. Miliaria and post miliarial hypohidrosis are well documented common causes of military disability. This study was designed primarily to determine the true prevalence of neomycin sensitivity in the general population since a high level of sensitivity may preclude the common use of neomycin by military physicians.

#### RESULTS AND DISCUSSION OF RESULTS

A pretested clinical questionnaire was administered in standard fashion to each volunteer prior to patch testing. The prevalence



of contact sensitivity to standard patch tests in 1158 volunteers was nickel (5.7%), neomycin (1.1%), ethylenediamine (0.43%), and benzocaine (0.17%). All 12 neomycin positive volunteers were reactive on three separate occasions. Nine of 12 had positive use tests to neosporin cream or ointment. Ten of 12 neomycin positive volunteers gave a past history of using a neomycin containing topical antibiotic on a rash for one week or longer. A retrospective neomycin case control study matching each neomycin positive with three control subjects matched for age, race, and sex revealed only 6 of 36 controls with a similar positive history of neomycin use. ( $\chi^2=14.4$ ,  $p>.001$ ). The relative risk of neomycin sensitivity increases thirteen-fold if it was previously used on a rash for 1 week or longer.

There was a marked difference in nickel sensitivity between men (1.1%) and women (9%). A history of pierced ears, earlobe rash, and jewelry rash all correlated well with nickel sensitivity. Thirty-five of 67 women (52%) were "nickel positive" who answered yes to the above three risk factors while only 2 of 164 (1.2%) women had a positive nickel patch test who answered no to the same three risk factors (relative risk = 43). The relative risks for a positive nickel patch test in women when history positives are compared to history negatives are jewelry rash (4.8X), ear lobe rash (7.3X), and pierced ears (4.6X).

#### CONCLUSIONS

The prevalence of neomycin patch test sensitivity in the general population was determined to be 1.1%. This low prevalence of sensitivity does not preclude the use of neomycin containing topical antibiotics. The twelve sensitive patients experienced erythema and pruritus 1 to 4 days after neosporin use. The pruritus disappeared in 2 to 3 days after they used topical steroid therapy. Thus, a sensitive patient experiences a little discomfort for only a few days.

#### RECOMMENDATIONS

The risk of contact sensitivity is extremely small when neomycin containing antibiotics are used as indicated.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6095	77 10 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62172A	3M162172A810	00	010			
<del>XXXXXXXXXX</del>	62772A	3M762772A810	00	010			
<del>XXXXXXXXXX</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Microbial Interactions on Healthy and Infected Skin of Soldiers							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
010100 - Microbiology, 003500 - Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 04		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE		EXPIRATION:		PRECEDING			
B. NUMBER*				FISCAL YEAR		61	
C. TYPE Not Applicable		D. AMOUNT:		CURRENT		2.2	
E. KIND OF AWARD		F. CUM. AMT.				96	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME* Letterman Army Institute of Research				NAME* Letterman Army Institute of Research			
ADDRESS* Presidio of San Francisco, CA 94129				ADDRESS* Department of Dermatology Research			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME* Keith, William Jr., CPT, MS			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5455			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				POC: DA			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Normal Flora; (U) Skin Ecology; (U) Microbiology; (U) Human Skin; (U) Human Volunteers; (U) Pyoderma; (U) Animal Models							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) One objective is to study bacterial skin infections (pyoderma-a disease that can occur in epidemic proportions among combat personnel) with the use of animal models in an effort to develop prophylactic measures against pyoderma which are presently not available. The role of normal cutaneous flora in host resistance against microbes causing epidemic skin infections in soldiers will be investigated. Characteristics of the skin and its environment which affects its susceptibility to these microorganisms will be studied.</p> <p>24. (U) Bacteria isolated from active lesions will be used in these studies. Animal models will be developed to determine the pathogenesis, prevention, and prophylaxis of pyoderma. By identifying and quantitating normal cutaneous microorganisms on healthy and infected skin of soldiers, topical bacterioprophyllactic measures will be developed to augment natural resistance.</p> <p>25. (U) 76 10 - 77 09. With the arrival of a new investigator, the major effort has been to develop protocols: a "Type Protocol" to sample the skin flora of volunteers, a study protocol to examine bacterioprophyllaxis and bacteriotherapy, and another study protocol in which animal models are used to investigate streptococcal and staphylococcal pyoderma await approval. The microflora of the foot before and after immersion in swamp water was determined, and the predominant bacteria isolated after immersion were gram negative cocci. A bacitracin negative mutant of <i>Bacillus licheniformis</i> ATCC 10716 was isolated, and in vitro it inhibited various skin staphylococci and a diphtheroid.</p>							

# ABSTRACT

PROJECT NO. 3M762772A810 Military Skin Diseases  
WORK UNIT NO. 010 Microbial Interactions on Healthy and  
Infected Skin of Soldiers

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Sampling of microorganisms from human skin
- STUDY NO. 2 The survival of bacilli, streptomyces, and other bacteria upon human skin
- STUDY NO. 3 Animal models for studying the pathogenic mechanisms of bacterial pyodermas

STUDY NO. 1 A pilot study was done to determine the extent, if any, of the participation of cutaneous microflora in warm water immersion foot, a disease which severely affects the mobility of combat foot soldiers. Bacteriological specimens were taken before and after each volunteer was subjected to experimental prolonged water immersion for 74 hours. Results indicated that the colony-forming-units of bacteria per square centimeter did not change significantly; however, the microflora which was gram positive before the experiment was gram negative afterwards. Vesicle fluid was generally found to be sterile. Cutaneous microflora may not play a dominant role in this disease, but the evidence remains equivocal.

STUDY NO. 2 To examine the role of antibiosis in microbial colonization of the skin, a nitrosoguanidine-derived antibiotic negative mutant of bacitracin-producing Bacillus licheniformis ATCC 10716 was allowed to interact in vitro by using the EcoloGen with Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus saprophyticus and a diphtheroid. The result was that these microorganisms were somewhat retarded in growth but to a lesser degree than that found with the parent ATCC 10716.

STUDY NO. 3 To investigate pyoderma, a disease that occurs in epidemic proportions among combat soldiers, a study protocol has been developed and is awaiting approval. With the use of bacteria isolated from active lesions, animal models will be developed to determine the pathogenesis, prevention, and prophylaxis of pyoderma.



## BODY OF REPORT

WORK UNIT NO. 010

Microbial Interactions on Healthy and  
Infected Skin of Soldiers

STUDY NO. 1

Sampling of microorganisms from human  
skin

### PROBLEM

Immersion foot is a disease which dermatologists have observed in combat soldiers. In support of a field study lasting 74 h which was being conducted, we took bacteriological specimens before the experiment was initiated and after the experiment was terminated to determine the extent that the microflora played in inducing immersion foot.

### RESULTS AND DISCUSSION OF RESULTS

The "before specimens" on cultivation grew predominantly gram positive cocci and diphtheroids. A dermatophyte was initially cultured from one volunteer while none was cultured after the experiment. Although a higher bacterial density was found after the experiment, the difference observed was not significant. Small vesicles were observed on all three volunteers' ankles and lower legs; cultures of the fluid from some of these vesicles were sterile. One of the volunteers had a small pustule which, on culturing, grew a coagulase positive Staphylococcus aureus. Most of the bacteria isolated after the experiment were gram negative cocci. It appears that the source of these bacteria was the water outdoors in which the volunteers were immersed to induce immersion foot.

### CONCLUSIONS

Although a cause-effect relationship regarding the role of cutaneous microflora in water and water immersion foot was not established, the evidence we have collected does not discount the possibility that bacteria play a role in this disease. We wish to state at this time that the evidence we have collected is insufficient to document the implication.

### RECOMMENDATIONS

Future studies are needed to determine the role of selected cutaneous pathogens on water immersion foot.

### PUBLICATIONS

None

PROBLEM

One major attribute for a candidate bacterioprophyllactic or bacteriotherapeutic agent is the ability to survive and grow on the skin temporarily yet for a sufficient duration to be effective against pathogenic streptococci and staphylococci colonizing a soldier's skin. Although we previously observed that a bacitracin-producing *Bacillus* survived much better than two strains of the species which lack antibiotics, the results might have been due to factors other than antibiotic production. Thus, the first task was to find two closely related strains of a bacterium of which only one was able to synthesize an antibiotic.

RESULTS AND DISCUSSION OF RESULTS

With n-methyl-n'-nitro-n-nitrosoguanidine as the mutagenic agent, a bacitracin-negative isolate of *Bacillus licheniformis* ATCC 10716 was obtained. Within the EcoloGen multiple-chambered diffusion vessel, used for batch cultures, the mutant (10716-M) interacted with representative skin isolates of *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Micrococcus luteus*, and a diphtheroid. Compared with our previous data, 10716-M seemed to retard somewhat the growth of the cocci and diphtheroid but, after 32 hours of incubation, it retarded it to a lesser degree than that observed with its parent ATCC 10716.

CONCLUSIONS

Strain 10716-M, although lacking bacitracin, still has a small antagonistic effect against skin flora when tested in broth culture.

RECOMMENDATIONS

The survival kinetics of 10716-M on the skin of volunteers should be examined and the results should be compared with the previous data using ATCC 10716. This will help determine whether or not antibiosis is important in the survival of skin-borne microorganisms.

PUBLICATIONS

None

### PROBLEM

Bacterial skin infections are of military interest because they occur in epidemic proportion among combat personnel and thereby reduce combat effectiveness. The exact extent of the combat man-days lost from pyoderma is unknown due to the unavailability of records; however, in one survey 18% of the soldiers fighting in the paddies in the MeKong Delta of Vietnam were infected. Since there is no currently acceptable preventive measure against streptococcal-staphylococcal skin disease of combat soldiers, a protocol has been developed to study bacterial skin infection (pyoderma) by using animal models.

### RESULTS AND DISCUSSION OF RESULTS

Strains of Group A beta hemolytic streptococci which were isolated from active pyoderma lesions of combat soldiers have been collected and subjected to preliminary characterization tests. These strains will be tested for their ability to cause pyoderma lesions on the skin of guinea pigs, hamsters, Mexican hairless dogs, albino rats, laboratory mice, and rabbits.

### CONCLUSIONS

These strains of Group A beta hemolytic streptococci will be tested in animals on approval of this study protocol.

### RECOMMENDATIONS

A standard animal model must be developed for bacterial skin infections, pyodermas, to be used for developing prophylactic measures which are presently unavailable.

### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8A. DISSEM INSTR* NL	8B. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3M162172A810	00	015			
b. CONTINUING	62772A	3M762772A810	00	015			
c. <del>XXXXXXXXXX</del> CARDS 114f							
11. TITLE (Precede with Security Classification Code)* (U) Development of Improved Insect Repellents for Military Use							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 005900 Environmental Biology; 002600 Biology							
13. START DATE 76 10		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT GRANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: Not applicable d. KIND OF AWARD:				18. RESOURCES ESTIMATE a. PRECEDING FISCAL YEAR 77 CURRENT 78 b. PROFESSIONAL MAN YRS 3 c. FUNDS (in thousands) 121 127			
19. RESPONSIBLE DOD ORGANIZATION NAME: Letterman Army Institute of Research ADDRESS: Presidio of San Francisco, CA 94129 RESPONSIBLE INDIVIDUAL NAME: Canham, J. E., COL., MC TELEPHONE: (415) 561-3600				20. PERFORMING ORGANIZATION NAME: Letterman Army Institute of Research Department of Tropical Medicine ADDRESS: Presidio of San Francisco, CA 94129 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: M. A. Moussa, LTC, MSC TELEPHONE: (415) 561-2421 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Rutledge, L. C., DAC Hooper, R. L., CPT, MSC POC:DA			
21. GENERAL USE Foreign Intelligence Not Applicable				22. KEYWORDS (Precede EACH with Security Classification Code) (U) Repellent; (U) Diethyl toluamide; (U) Mosquito; (U) Sand Fly; (U) Flea; (U) Tick; (U) Sensory Receptor; (U) Ultrastructure			
23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) The objective is to develop improved insect repellents for use by military personnel in areas or operational situations where effective disease vector control measures are impractical. Effective and long-lasting repellents are essential to insure combat effectiveness of units exposed to vector-borne diseases. 24. (U) Selected major disease vectors will be colonized; appropriate testing procedures will be developed; compounds and formulations will be screened and evaluated on a systematic basis in the laboratory and in the field; basic and related studies will be conducted concurrently. 25. (U) 76 10 - 77 09 Eleven vector species of mosquitoes, sand flies, fleas, and ticks were reared for repellent testing. New methodology was developed for quantitative evaluation of mosquito, flea and tick repellents in in vitro, animal, and human test systems. Candidate repellents synthesized by Stanford Research Institute, Inc., were screened for repellency to mosquitoes in the laboratory and for effectiveness against mosquitoes in the field. Inbreeding effects on the response of mosquitoes to repellents were studied. Chemosensory organs on mosquitoes and sand flies were studied with light and electron microscopy. New types of receptors were discovered on the <i>Aedes aegypti</i> tarsi and sand fly antennae.							

\* Available to contractors upon originator's approval

# ABSTRACT

PROJECT NO. 3M76227A810

Military Skin Disease

WORK UNIT NO. 015

Development of Improved Insect  
Repellents for Military Use

The following investigations have been conducted under this work unit:

STUDY NO. 1 Colonization of selected insect vectors of disease

STUDY NO. 2 Genetic aspects of mosquito repellency

STUDY NO. 3 Development of improved tick repellents for  
military use

STUDY NO. 4 Development of duration testing methods for  
mosquito repellents

STUDY NO. 5 Studies of repellent receptors in disease vectors

STUDY NOS. 1, 2, 3, 4, and 5. Eleven species of mosquitoes, sand flies, fleas and ticks were reared for repellent testing. New methodology was developed for quantitative evaluation of mosquito, flea and tick repellents in in vitro, animal, and human test systems. Selected conventional repellents were tested against fleas and ticks. A number of candidate compounds obtained from Stanford Research Institute and the U. S. Department of Agriculture were screened for repellency to mosquitoes. Inbreeding effects on the responses of mosquitoes to repellents were determined, and cross-tolerances of inbred mosquitoes to different repellents were studied. Chemosensory organs of mosquitoes and sand flies were studied by light and electron microscopy. New types of receptors were discovered on the tarsi of Aedes aegypti and the antennae of sand flies.

## BODY OF REPORT

WORK UNIT NO. 015

Development of Improved Insect  
Repellents for Military Use

STUDY NO. 1

Colonization of selected  
insect vectors of disease

### PROBLEM

Laboratory investigations for developing improved repellents against militarily significant disease vectors depend on the availability of an adequate stock of representative insect species for experimental use. Laboratory colonies provide an economical in-house resource for repellent bioassay in lieu of travel to distant geographical regions where important species are known to occur. This study encompasses research relating to developing methods for the laboratory production of experimental insects required for the repellent development program. Emphasis is on the development of practical, efficient methods for mass-producing selected, representative species which have military medical importance.

### RESULTS AND DISCUSSION OF RESULTS

Several species of mosquitoes were maintained throughout the year and reared in large numbers sufficient for the support of repellent studies. These species included known vectors of malaria, viral encephalitis, dengue, yellow fever, and filariasis as well as major pest species. The colonies continue to represent the most diversified collection of mosquito species of medical importance available for repellent testing in any laboratory. Substantial improvements have been made in the rearing of mosquito colonies which contributed to enhanced productivity and ease of handling. A new larval feeding regimen was introduced and adopted for routine use for the malathion-resistant strain of Anopheles quadrimaculatus. It involved feeding early instar larvae with an infusion of 1:1 liver extract and brewer's yeast for the first 2 days to be followed by a 1:1 mixture of 40% hog supplement and floating catfish chow. This diet helped improve larval development and survival.

Special devices were fabricated for restraint of animals being used for blood feeding adult mosquitoes. A simple wooden rabbit restrainer helped position the animal on top of the mosquito cage. The device, therefore, permitted the personnel to attend to other tasks in the meantime.

Efforts to design an in vitro bloodfeeding system suitable for use in the LAIR insectary continued. The first apparatus did not prove satisfactory. A much simpler substitute feeder system has been designed and is currently under test. If it proves to be satisfactory, this new system will be adopted for routine use in lieu of animal donors.



A colony of the hard tick Derma-centor variabilis (Say), a major vector of Rocky Mountain spotted fever and tularemia, was successfully mass reared. Also, two new colonies of soft ticks, Ornithodoros hermsi Wheeler, Hermes and Meyer and Ornithodoros parkeri Cooley were initiated. These two species are vectors of relapsing fever. The acquisition of these ticks constitutes a significant addition to the species of vectors available for use in the repellent testing program.

A colony of the flea Diamanus montanus Baker, which is a known vector of sylvatic plague, was successfully mass reared for use in repellent tests. Rearing techniques were improved by using ground corn cob in lieu of filter paper as a substrate in plastic jars. The colony of Hoplopsyllus anomalus Baker initiated at the same time was lost due to an incubator failure.

The colony of the phlebotomine sand fly, Lutzomyia longipalpis (Lutz and Neiva), a major vector of leishmaniasis in South America, has been successfully maintained since its initiation in May 1975. Because of their relatively narrow tolerances, low fecundity and long life cycle, the phlebotomine sand flies are among the most difficult to rear in large numbers in the laboratory. A major advance in sand fly rearing techniques was the development of small (15x17x17 cm) solid plexiglass cages. These cages help retain the proper level of humidity, minimize escape, and improve the handler's ability to see the flies.

#### CONCLUSIONS

A large battery of test species was successfully maintained or mass reared for use in repellent research. Significant advances were made in laboratory production methods for mosquitoes, ticks, fleas, and sand flies.

#### RECOMMENDATIONS

Research on insect rearing methods should be continued with emphasis on economy of operation and requirements for the repellent program. Because of the military significance of certain species of mosquitoes, emphasis should be placed on improving mosquito rearing techniques. The sand fly rearing methods should be improved further.

#### PUBLICATIONS

RUTLEDGE, L. C., R. K. SOFIELD, and G. N. PIPER. Rapid counting methods for mosquito larvae. Mosq News 36: 537-540, 1976.



PROBLEM

The USAMRDC supports an extensive program of research on chemical repellents for the protection of military personnel from mosquito-borne diseases. Investigators evaluating candidate repellents frequently have reported disparate or conflicting results. It is believed that genetic factors relating to the test mosquitoes may account, in part, for those discrepancies. The present study provides for a quantitative assessment of the relative importance of environmental and genetic factors in determining the responses of mosquitoes to repellents and for the development of genetically homogeneous strains of the yellow fever mosquito, Aedes aegypti (Linnaeus) for evaluation as standardized repellent test strains.

RESULTS AND DISCUSSION OF RESULTS

Five inbred strains of Aedes aegypti derived earlier in the study from the MASAKA (diethyl toluamide-sensitive) and MOYO INDOOR (diethyl toluamide-tolerant) strains were tested to determine the effects of inbreeding on the sensitivity of the daughter strains to diethyl toluamide (deet). The results of these tests indicated that inbreeding substantially reduced the level of tolerance to deet in both the MASAKA and the MOYO INDOOR strains. Intrastrain variability in deet-sensitivity was not reduced by inbreeding. The phenomenon of inbreeding depression, as typified by the foregoing results, is antipodal to heterosis in polygenic systems of inheritance. Accordingly, tolerance to deet in Aedes aegypti has an heterotic component. In such cases, it has been recommended that the  $F_1$  hybrids of 2 inbred strains be used in bioassay work, since such hybrids are both heterozygous and genetically uniform.

Four inbred strains of Aedes aegypti were tested to determine their respective levels of sensitivity to deet and ethyl hexanediol. The correlation of levels of sensitivity to deet with levels of sensitivity to ethyl hexanediol was not statistically significant. These results indicated that levels of sensitivity to deet and ethyl hexanediol in Aedes aegypti are determined by separate physiological pathways in the mosquito. From a practical standpoint, these findings mean that one repellent could be substituted for the other if repellent tolerance were encountered in the field.

The foregoing inbred strains of Aedes aegypti were morphologically and biologically compared to determine whether their respective levels of sensitivity to deet and ethyl hexanediol could be correlated with any external or measurable characters that might serve as genetic markers.

Although the several inbred strains differed significantly in morphology (size, color of the integument, color and pattern of the integumental scales, etc.) and biological characteristics (clutch size, hatching period, hatch rate, larval survival, pupal survival, adult male and female longevity, etc.), specific indicators of levels or degrees of sensitivity or tolerance to deet and ethyl hexanediol were not found. This line of investigation has been extended under Study No. 5 to include electron microscopic studies of the chemosensory receptors of the inbred strains and the parent MASAKA and MOYO INDOOR strains.

It is possible, in principle, for cross-resistance between insecticides and repellents to develop in populations of mosquitoes in the field. Genetic linkages of this type would be of practical importance in the selection of repellent compounds for use against insecticide-resistant populations of vector species. In the present study, comparative data were obtained on the respective tolerances of 11 laboratory strains of Anopheles albimanus, Anopheles stephensi, Anopheles quadrimaculatus, Aedes aegypti, and Culex pipiens to deet and malathion and on the respective tolerances of 7 strains of Anopheles stephensi, Anopheles quadrimaculatus, Aedes aegypti and Culex pipiens to deet and DDT. The results obtained in the study do not support the hypothesis that genetic linkages between the military standard repellent (deet) and organophosphorus insecticides (malathion) or chlornated hydrocarbon insecticides (DDT) are likely to occur in the field. No such linkage was detected in the several laboratory strains (field isolates) of mosquitoes available for study at LAIR.

New compounds synthesized by Stanford Research Institute, under contract with USAMRDC, and new candidate repellents obtained from the U. S. Department of Agriculture were tested for their repellency to mosquitoes. To date, approximately 100 compounds have been tested for repellency to Aedes aegypti. The median effective dosages of these chemicals ranged from 0.8  $\mu\text{g}/\text{cm}^2$  upward, including several compounds which had no detectable effect. Approximately one-third of the chemicals tested were just as or more repellent than deet. Thus, our baseline data on this species effectively cover its entire known range of genetically determined avoidance responses to chemicals. The correlation of these responses with chemical structure and properties is currently being studied at Stanford Research Institute.

Since one of the objectives of the present study is to assess the relative importance of hereditary and environmental factors in the feeding behavior of Aedes aegypti, the probing responses of that species to nine persons were critically studied. Some investigators have thought that mosquitoes may be more inclined to feed on one person than another and that such mosquito "preferences" might tend to counteract the repellent effect of deet on the skin of the preferred individual. While some investigators have reported experimental confirmation of the supposed preferences of mosquitoes for certain individuals, others have been unable to arrive at that result.

The experimental design used in the present study incorporated more advanced bioassay procedures than had been previously applied in this area of research. Probit analysis of the results obtained indicated that the mosquitoes tested did not respond preferentially to any of the nine test subjects. It is believed that previous investigators have mistaken non-appetential behavior on the part of the mosquito for an expressed "non-preference" for the test subject.

#### CONCLUSIONS

The avoidance responses of mosquitoes to repellents are determined by complex interactions of genetic and non-genetic factors. The genetic factors involved include a polygenic component which can be demonstrated by inbreeding techniques. Different genetic systems are involved in determining the responses to different repellents. The behavioral responses of mosquitoes to repellents are not genetically linked with their physiological responses to insecticides. The responses to repellents are not modulated by differential "preferences" for individual human hosts.

#### RECOMMENDATIONS

Levels of sensitivity of the hybrids of inbred strains of Aedes aegypti to deet and ethyl hexandiol should be measured, and the suitability of the hybrids for use as standardized bioassay test subjects should be determined. Heritability of the deet-sensitivity and the ethyl hexanediol-sensitivity traits should be determined through tests of the  $F_1$  hybrids and the backcrosses. Genetic analysis of the inbred strains should be extended to include the repellents.

#### PUBLICATIONS

RUTLEDGE, L. C., M. A. MOUSSA, C. A. LOWE and R. K. SOFIELD. Comparative sensitivity of mosquito species and strains to the repellent diethyl toluamide. J Med Entomol 14:536-541, 1978.

STUDY NO. 3

Development of improved tick repellents for military use

#### PROBLEM

Military personnel are often required to enter areas which are endemic for tick-borne diseases. It is often impracticable to treat such areas with an effective insecticide to control the tick population prior to the time that troops are required to enter the region. Protection of troops from tick-borne diseases in such situations depends on the use of effective tick repellents. The current standard repellents, M-1960 for clothing application and deet for topical application, are not totally effective against ticks. There is a current need for longer-lasting repellents having high intrinsic repellency for ticks.



## RESULTS AND DISCUSSION OF RESULTS

New methods for testing tick repellents were developed for use in the study in lieu of conventional test methods, which are, at best, only semiquantitative. (a) Median Effective Dosage ( $ED_{50}$ ): Suckling mice were treated by dipping in ethanol (control) and serial dilutions of the test repellent in ethanol (repellent treatments). The mice were allowed to dry for 30 minutes, after which 75 ticks were allowed access to them on a "free-choice" basis for an additional 3 1/2 hours. The (four-hour)  $ED_{50}$  of the repellent was determined by probit analysis of the numbers of ticks attached or present on the mice at that time. (b) Effective Halflife: In this test, all the mice (except the control) were treated with the same dilution of repellent, but the treatments were made at successive intervals of time prior to the test. The effective halflife of the repellent was calculated from the numbers of ticks attached or present on the mice when the test was terminated. The repellent effect was defined in terms of probability units (probits) for the halflife calculation, in accord with the  $ED_{50}$  calculation.

The military standard repellent, diethyl toluamide (deet), and 3 possible<sup>R</sup> alternatives, ethyl hexanediol, dimethyl phthalate, and Indalone<sup>R</sup>, were evaluated. Although these 4 compounds are accepted repellents which are sold commercially and are known to be effective against ticks, they had not been critically tested and compared by modern methods. The  $ED_{50}$  and the effective halflife were determined for each of the 4 compounds, using the nymphal stage of the eastern dog tick, Dermacentor variabilis. This species is the primary vector of Rocky Mountain spotted fever in the central and eastern U. S.

The  $ED_{50}$  of deet, ethyl hexanediol, dimethyl phthalate and Indalone<sup>R</sup> for D. variabilis were 0.12, 0.41, 0.33, and 0.11% respectively. Thus, deet and Indalone<sup>R</sup> were equally effective, on a gram-for-gram basis, in repelling the test insect. Dimethyl phthalate was less effective than deet or Indalone<sup>R</sup> but more effective than ethyl hexanediol.

The effective halflives of deet, ethyl hexanediol, dimethyl phthalate, and Indalone<sup>R</sup> for protection against D. variabilis were 7.5, 8.4, 7.3, and 6.6 hours respectively. Thus, the repellent effects of ethyl hexanediol applications were more persistent than those of deet and dimethyl phthalate applications, and the repellent effects of Indalone<sup>R</sup> applications were less persistent than those of deet and dimethyl phthalate applications.

The methods used in this study permit, for the first time, measurement of the rate of decay separately from dosage effects in studies relating to persistence of repellents on the skin. These two distinct processes were confounded in conventional repellent tests because the measure of persistence used (protection time) was dose-dependent. Since the effective halflife of a repellent is a measurement of rate of decay, it is, within limits, independent of the dosage of repellent applied.



The limits referred to are those applicable to bioassay techniques in other fields of study, viz., 0-100% effect. Adoption of bioassay techniques in the study has provided quantitative tick repellent test results for the first time.

#### CONCLUSIONS

Diethyl toluamide is superior to ethyl hexanediol, dimethyl phthalate, and Indalone<sup>R</sup> as a repellent for use against D. variabilis because of its low ED<sub>50</sub> (0.12%) and favorable persistence qualities (effective half-life = 7.5 hours). Ethyl hexanediol, dimethyl phthalate and Indalone<sup>R</sup> are acceptable alternatives to diethyl toluamide, provided that they are applied more heavily (ethyl hexanediol, dimethyl phthalate), or frequently (Indalone<sup>R</sup>).

#### RECOMMENDATIONS

The study should be extended to include (a) larval and adult stages of D. variabilis, (b) the opposing family of ticks (Family Argasidae), and (c) selected new repellent compounds which may be superior to deet.

#### PUBLICATIONS

None

STUDY NO. 4

Development of duration testing  
methods for mosquito repellents

#### PROBLEM

While many chemicals are known to repel mosquitoes effectively when applied to the skin at practical dosages, relatively few persist on the skin for extended periods of time after application. Persistence is critical to the military application, since military operations may require continuous exposure of personnel to mosquitoes for prolonged periods of time. Conventional methods are inadequate for unequivocal determination of the persistence properties of new mosquito repellents. Quantitative, statistical methods based on modern bioassay techniques are critically needed in the repellent development program supported by USAMRDC.

#### RESULTS AND DISCUSSION OF RESULTS

In vitro and animal test systems were developed as alternatives to the use of human test subjects in primary screening and testing programs. In the in vitro test system natural loss of repellent from

the skin was simulated in the laboratory with a set-up which incorporated a membrane (representing the skin) overlying a gel (representing the flesh). Repellent-treated membranes were incubated for varying periods of time in the system and subsequently transferred, together with an untreated control membrane, to an in vitro mosquito blood-feeding system for bioassay of the repellent residues. The system provides for the regulation of the rates of evaporation and absorption of repellents by choice of membrane (reconstituted collagen, goldbeater's skin, etc.), choice of gel (dextran, agar, etc.), and by regulation of moisture, temperature, air movement, and other conditions. Sweating can be simulated by providing for exudation or condensation of moisture at the membrane surface. It is anticipated that appropriate adjustments and substitutions in the in vitro test system will bring its "readings" into close correspondence with the "readings" obtained from the equivalent test system utilizing human test subjects.

The animal test system developed in the study utilizes suckling mice because of their small size, lack of hair, immobility, and low cost. The animals were treated at fixed intervals of time with a dilute solution of repellent in ethanol. Treatment was by brief immersion, in the same manner that sheep and cattle are treated with insecticides. Subsequently, the repellent-treated mice and an ethanol-treated control mouse were exposed to a test population of mosquitoes for bioassay of the repellent residues. As in the case of the in vitro repellent test system, it is anticipated that appropriate adjustment of the white mouse test system will bring its readings into close correspondence with those obtained from the equivalent human test system.

New procedures for advanced testing of repellents on human test subjects in the laboratory and the field were developed. In the laboratory test method, a dilute solution of repellent was applied at fixed intervals of time to circular test areas outlined on the flexor region of the forearm. The treated areas were subsequently exposed, together with an ethanol-treated control area, to a test population of mosquitoes for bioassay of the repellent residues.

The field test method was validated in trials of diethyl toluamide and ethyl hexanediol against Aedes dorsalis at Skaggs Island, CA, in August and September 1977. In the field test method, a dilute solution of repellent was applied at fixed intervals of time to the two forearms and one of the lower legs or to the two lower legs and one of the forearms, the remaining member being selected at random for treatment with ethanol only as a control. Subsequently, the test subjects entered a mosquito-infested area and exposed the forearms and lower legs to the mosquitoes. Mosquitoes were collected separately from the exposed limbs for subsequent counting and identification.

The newly developed test methods were designed specifically to measure the effective halflives of mosquito repellents. They permit,

for the first time, measurement of the rate of decay separately from dosage effects in studies relating to the duration of mosquito repellents on the skin. These two distinct processes were confounded in conventional repellent tests because the measure of persistence used (protection time) was dose-dependent. Since the effective half-life of a repellent is a measure of rate decay, it is, within limits, independent of the dosage of repellent applied. The limits referred to are those applicable to bioassay techniques in other fields of study, viz., 0-100% effect in probability units. Adoption of bioassay techniques in the study has provided quantitative mosquito repellent test results for the first time. The effective half-lives estimated for diethyl toluamide and ethyl hexanediol in the Skaggs Island trials (9.6 and 12.4 hours, respectively) are undoubtedly the most meaningful measures of the persistence properties of these two important repellents that have been made to date, even though the materials have been in use since the 1940s (ethyl hexanediol) and 1950s (diethyl toluamide).

#### CONCLUSIONS

A diversified battery of new test methods was developed for evaluating the persistence properties of mosquito repellents. These included in vitro and animal test methods for primary screening and testing in the laboratory as well as human test methods for advanced testing of repellents in the laboratory and the field. The new methods achieve a major advance in repellent testing methodology by incorporating standard bioassay techniques into the experimental design and providing for measurement of rate of decay separately from dosage effects for the first time.

#### RECOMMENDATIONS

The laboratory test methods described should be further refined and finalized and the field test method should be further validated. The new test methods should be adopted for use at LAIR and elsewhere, and the older test methods should be discontinued as obsolete.

#### PUBLICATIONS

RUTLEDGE, L. C. Incubation and prepatent periods of Plasmodium vivax. Trans Roy Soc Trop Med Hyg 71:451, 1977.

STUDY NO. 5

Studies of repellent receptors in disease vectors

#### PROBLEM

The development of improved repellents for military use requires an understanding of the mode of action of these chemicals through the vector's chemosensory pathways. The current repellent-synthesis



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program supported by this command emphasizes the synthesis of volatile chemicals in the assumption that vectors perceive repellents through their olfactory receptors. The role of other sensory receptors has been either neglected or not fully appreciated. The purpose of this study is to locate and describe the sensory receptors of selected disease vectors, and to correlate receptor type and/or distribution with the vector's response. This information may provide significant leads to the chemist in selecting chemical properties most likely to yield the best repellents, and may also provide a method to predict the species response to different repellents on the basis of its sensory complement.

#### RESULTS AND DISCUSSION OF RESULTS

Both scanning and transmission electron microscopy have been used to examine sense organs on the antennae and tarsi of the sand fly, Lutzomyia longipalpis. The antennae are primary loci of olfactory receptors and the tarsi are the primary loci of the gustatory receptors. A morphologically unique receptor was found on the sand fly antenna. This receptor is a small (length 2.7  $\mu$ ) thorn-shaped, grooved peg encircled by 8 microtrichiae. The grooved peg has an internal structure similar to that of the grooved pegs on mosquito antennae. However, those on the mosquito lack the associated microtrichiae. On the mosquito, grooved pegs are known to respond to lactic acid. The sand fly may be repelled by chemical compounds that repel mosquitoes, since they possess similar sense organs. Six to ten thick-walled or gustatory hairs were found on the foretarsus of the sandfly. These hairs are exceptionally few in number, compared with those found on other biting Diptera. The sand fly is a small, delicate fly and it is possible that it relies more on olfactory and/or thermal stimuli rather than gustatory stimuli to find its host.

Light and electron microscopy were used to demonstrate the presence of olfactory hairs on the tarsi of the mosquito, Aedes aegypti. Olfactory hairs have not been previously reported on the tarsus of any arthropod. The olfactory hairs externally resemble the more numerous gustatory hairs. Both are about 35  $\mu$  in length, but transmission electron microscopy reveals that these hairs are definitely thin-walled or olfactory. Since the mosquito's tarsus is covered with scales, these may afford protection to the delicate thin-walled olfactory hairs. Olfactory hairs on the tarsi may explain how mosquitoes are able to respond to a repellent immediately before they alight on a repellent-coated surface.

#### CONCLUSIONS

Morphological examination of chemoreceptors on disease vectors of military medical importance is providing insight into the vector's response to repellents. However, vector species studied to date are too few to determine exactly which receptors are crucial to the vector's being repelled from its host.

#### RECOMMENDATIONS

The sensory morphology of numerous diverse medically important disease vectors should be examined and their morphology correlated with the vector's known behavioral response to different repellents.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DES'N INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62172A	3M162172A810		00		016	
<del>B. SECONDARY</del>	<del>62772A</del>	<del>3M762772A810</del>		<del>00</del>		<del>016</del>	
<del>C. TERTIARY</del>	<del>CARDS 114f</del>						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Diagnosis and Prevention of Leishmaniasis in Military Personnel							
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0010100 Microbiology; 002600 Biology							
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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E. TYPE: Not applicable		F. AMOUNT:		CURRENT YEAR		163	
G. KIND OF AWARD:		H. CUM. AMT.		78		3.5	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not applicable				ASSOCIATE INVESTIGATORS			
				NAME: Childs, G.E., CPT., Luzzio, A.J., DAC,			
				NAME: Wilson, H.R., DAC, & Rutledge, L.C., DAC			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Antigen; (U) Diagnosis; (U) Immunity; (U) Disease Vector; (U) Leishmania; (U) Serology; (U) Epidemiology							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective is to develop improved methods for the diagnosis and prevention of cutaneous leishmanial infections encountered by service personnel deployed or stationed in endemic areas overseas. The absence of protective vaccines and the lack of satisfactory treatment renders these studies essential to the protection of the health of the field soldier.</p> <p>24. (U) Mass culture of <u>Leishmania</u> will be instituted to provide sufficient cells for biochemical isolation of cellular components. Immunochemical analysis will be conducted to define components which offer greater sensitivity and specificity for utilization in serodiagnosis and skin testing methods. In vivo immune responses to purified components and in vitro immunochemical reactions will be conducted to characterize humoral and cell-mediated mechanisms and to evaluate antigens for their potential protective value against <u>Leishmania</u>.</p> <p>25. (U) 76 10 - 77 09 Fractionation of <u>L. braziliensis</u> by differential centrifugation and chromatography produced a component which showed high antigenic reactivity in passive hemagglutination tests with sera from subjects infected with American leishmaniasis. Rabbits immunized with cell extracts of <u>Leishmania</u> demonstrated complement fixing antibody 30 days after infection and precipitating antibody after 31-138 days. A rise in antibody hemolytic for sheep red cells demonstrated Forssman antigen in <u>Leishmania</u>. Guinea pigs sensitized with <u>L. enriettii</u> showed specific delayed skin responses when tested with several leishmanin preparation's.</p>							

# ABSTRACT

PROJECT NO.	3M762772A810	Military Skin Disease
WORK UNIT NO.	016	Diagnosis and Prevention of Leishmaniasis in Military Personnel

The following investigations have been conducted under this work unit:

STUDY NO. 1 Serodiagnosis of American leishmaniasis

STUDY NO. 2 Improved skin test antigen for the diagnosis and prognosis of leishmaniasis

STUDY NO. 3 Immune mechanisms in leishmaniases

STUDY NO. 1. A system was established which utilized insect cell culture media in cell culture flasks and roller bottles for the routine mass cultivation of low passage leishmanial promastigotes. Promastigotes of Leishmania braziliensis were sonicated and antigenic fractions, obtained by differential centrifugation and gel diffusion chromatography, were evaluated by passive hemagglutination (PHA) tests for detecting leishmanial antibodies in sera from confirmed leishmania cases. The PHA test, which employed an antigen derived from the supernatant of a high speed centrifugation, was able to detect leishmanial antibodies in 4 of 6 (67%) patients with early infections of cutaneous leishmaniasis. In addition to the PHA, the feasibility of an enzyme-linked immunosorbent assay (ELISA) for the detection of leishmanial antibodies was investigated.

STUDY NO. 2. Studies were conducted for the preparation, standardization, and evaluation of skin testing antigens for the diagnosis and prognosis of leishmaniasis. Skin testing experiments have indicated that there are no differences in delayed hypersensitivity responses between groups of guinea pigs sensitized with amastigotes or promastigotes. Likewise, there is little variation between responses induced by amastigote- or promastigote-derived antigens. Leishmanin preparations of Leishmania enriettii and L. braziliensis tested in the L. enriettii-guinea pig system showed positive delayed responses with no responses in unsensitized controls. A leishmanin skin testing antigen was produced from promastigotes cultured in media which lacked ingredients which would limit its acceptability for human use.

STUDY NO. 3. The formation of circulating immune complexes in hamsters infected with Leishmania mexicana was indirectly demonstrated. Anti-complementary activity (AC) occurred more frequently in hamsters during early infection than in late infection

or in normals. A greater number of sera were complement fixing (CF) antibody positive in L. braziliensis than in L. mexicana infection, with CF titers ranging from 1:4 to 1:64. The formation of immune complexes offers a rational explanation for the transient nature and elusiveness of leishmanial antibody. Efforts to produce high titer anti-leishmania sera in rabbits with antigenic preparations of L. mexicana consistently produced weak immune responses in these animals. However, L. mexicana antigens induced a rise in sheep red blood hemolysin and demonstrated the presence of Forssman antigen which may be important in species differentiation and host resistance.



## BODY OF REPORT

WORK UNIT NO. 016

Diagnosis and Prevention of  
Leishmaniasis in Military  
Personnel

STUDY NO. 1

Serodiagnosis of American  
leishmaniasis

### PROBLEM

Standard serological techniques lack specificity and sensitivity in leishmanial infections to detect low levels of specific circulating antibodies. Thus, infections are not ordinarily diagnosed until a grossly observable lesion develops. Early diagnosis will permit early treatment and decrease serious complications from mucocutaneous or visceral involvement. This study was designed to develop a simple but effective serological test for use in detecting early pre-patent leishmanial infections among military personnel serving in, or returning from, endemic areas of this disease.

### RESULTS AND DISCUSSION OF RESULTS

Initial attempts to adapt a series of insect cell culture media for the mass cultivation of leishmanial parasites were often unsuccessful due to the failure of amastigotes, at a low density, to adapt to the media and to transform to promastigotes. Fungal contamination of the cultures was frequently experienced. These problems were resolved by initiating leishmanial growth in small volume cell culture flasks and roller bottles prior to inoculation of mass culture flasks. This procedure facilitates the adaptation and transformation of amastigotes and decreases the frequency of contamination. Efficiency of harvest was significantly improved by the use of continuous flow centrifugation. This system has made it possible to collect routinely 10 to 12 ml of packed promastigotes per 6 liter flask. Although higher yields may be possible, cells were harvested early in the log-growth phase to insure collection of a greater number of viable promastigotes.

Sonicated homogenates of L. braziliensis promastigotes were fractionated by differential centrifugation and gel diffusion chromatography. Ten antigenic components were isolated and evaluated in a passive hemagglutination (PHA) test which used sensitized, glutaraldehyde-fixed human Type O negative erythrocytes to detect leishmanial antibodies in sera from experimental animals and humans with confirmed cases of leishmaniasis.

Gel diffusion chromatography of supernatants obtained after relatively low speed centrifugation (3,000 g for 30 min) revealed



a prominent high molecular weight (MW) peak and two lower MW peaks. PHA determinations with sera from immunized rabbits and chickens and from infected hamsters showed the highest titers with fractions eluted within the first low MW peak.

Higher speed centrifugation (20,000 or 30,000 g for 1 h) was used to separate soluble from insoluble portions of sonicated promastigotes. The soluble portion was used as an antigen in the PHA test for the assay of leishmanial antibodies in a series of human patients with confirmed cutaneous leishmaniasis. Titers were noted in the sera of 4 out of 5 patients. These included 3 patients with relatively early infections including one of 1 1/2 months (1:32) and one of 2 months (1:16). The third early infection (2 months' duration) showed no significant titer. Titers were also detected in sera from 2 patients with chronic infections, one with mucocutaneous leishmaniasis (1:64) and another with diffuse cutaneous leishmaniasis (1:16).

Three additional series of sera from patients with simple cutaneous infections were also tested. The first series of 7 sera, collected from a patient at periodic intervals within a 10-month period, showed a consistent rise in titer from 1:4 to 1:32. The first significant titer was noted 4 months after diagnosis. By contrast, the second series of 11 sera collected during an 18-month period showed a decrease in titer from 1:32 to <1:8. The third series collected over an 8-month period also showed a consistent decrease in titer. The results indicate that early leishmanial antibodies were detected with soluble antigen in the PHA of sera collected from 4 of 6 (67%) patients. Among uninfected humans, only one of 28 serum samples showed a titer.

Supernatants from high speed centrifugation were further purified by passage through a gel diffusion chromatography column. Two protein peaks were observed and collected. Initial serological evaluation by the PHA test with immune rabbit sera indicated that the major antigenic components were associated with the higher MW peak.

Limited trials with soluble and insoluble L. braziliensis promastigote antigens and anti-L. braziliensis rabbit sera in an enzyme-linked immunosorbent assay (ELISA), in which a bound alkaline phosphatase conjugate was used, indicated that this method can be used to detect leishmanial antibodies. The test is being adapted for a microtiter system in which leishmanial antigen-antibody-bound conjugate may be assayed accurately colorimetrically to quantitate leishmania antibody in infected sera.

#### CONCLUSIONS

Routine large scale propagation of leishmanial parasites was achieved in a system which involves the successive use of cell culture flasks, roller bottles, and 6 liter flasks. The use of

continuous flow centrifugation enhanced the efficiency of the harvest. Leishmanial antigens were fractionated by differential centrifugation and gel diffusion chromatography and reactivity was demonstrated by the PHA test.

#### RECOMMENDATIONS

The soluble antigens derived from high speed centrifugation of sonicated promastigotes should be further purified by gel diffusion and ion exchange chromatography to eliminate proteins which may affect specificity and sensitivity of serological tests. Insoluble sediments should be evaluated in serological tests which do not require soluble antigens, such as the ELISA test, or subjected to protein solubilization techniques. Soluble antigens should be examined in a variety of in vitro serological procedures to evaluate their suitability for the detection of low-level circulating antibodies.

#### PUBLICATIONS

CHILDS, G. W., M. J. MC ROBERTS, and K. A. FOSTER. Partial purification of amastigotes from cutaneous lesions of American leishmaniasis. *J Parasitol* 62:676-679, 1976

STUDY NO. 2

Improved skin test antigens  
for the diagnosis and prognosis  
of leishmaniasis

#### PROBLEM

Leishmanial infections represent a group of parasitic diseases encountered by military personnel stationed in endemic areas. Immunity in leishmaniasis is conferred principally by cell-mediated immune responses. Skin testing procedures to demonstrate delayed hypersensitivity are important aids for diagnosis and prognosis. Although skin testing with leishmanin (phenolated suspension of *Leishmania* promastigotes) is widely and successfully used overseas, leishmanin is not approved or licensed for use in the U. S. This study involves the preparation, standardization, and evaluation of antigens which will be acceptable for skin testing of U. S. personnel.

#### RESULTS AND DISCUSSION OF RESULTS

Experiments were undertaken to compare and characterize hypersensitivity responses to amastigote and promastigote antigens in guinea pigs sensitized to infections of *Leishmania enriettii*. Infections were initiated by quantitated inocula of amastigotes and promastigotes. Antigens evaluated for potential use in skin testing included whole and sonicated amastigotes and promastigotes of *L. enriettii*, and *L. enriettii* and *L. braziliensis* leishmanin preparations. Appropriate

controls for media and diluents were incorporated. Neither immediate responses by 2 h nor Arthus reactions at 6 h were noted. Delayed hypersensitivity responses were elicited by 48 h. No major differences were noted between responses in guinea pigs with infections initiated with promastigotes or amastigotes, or between amastigote and promastigote derived antigens. Among the antigens tested, only the leishmanin preparations failed to elicit responses in control (unsensitized) animals. Hypersensitivity responses were also obtained in guinea pigs inoculated with amastigotes of L. braziliensis.

Current procedures generally utilized for the cultivation of leishmanial antigens include whole rabbit and human blood, and certain other blood products which render these antigens unacceptable for human use in the U. S. Efforts were therefore made to cultivate leishmanial parasites in a media which contains none of these questionable substances. Promastigotes of L. braziliensis were successfully propagated in a defined medium. Cells were collected, prepared as leishmanin, and stored at -20°C for eventual evaluation of efficacy, chemical composition, sterility, safety, and toxicity.

#### CONCLUSIONS

Results of skin testing experiments with leishmanial antigens indicated that there are no discernible differences in hypersensitivity responses between guinea pigs sensitized with either amastigotes or promastigotes. Likewise, responses induced by amastigote or promastigote derived antigens were similar. Leishmanin preparations of L. enriettii and L. braziliensis evaluated in the L. enriettii-guinea pig system elicited positive delayed skin responses. No responses were obtained in unsensitized controls. Guinea pigs sensitized with viable amastigotes of L. braziliensis developed delayed hypersensitivity-skin responses and may be suitable for evaluation of leishmanin skin testing antigens.

#### RECOMMENDATIONS

Leishmanin skin testing antigen produced from promastigotes cultured in a defined medium with fetal calf serum should be evaluated for its capacity to elicit delayed hypersensitivity responses in guinea pigs sensitized by an infection of L. enriettii. Subsequently, testing and evaluation for chemical composition, sterility, safety, and toxicity of these preparations should be undertaken to satisfy the requirements for clearance and approval for initial human testing.

#### PUBLICATIONS

None



PROBLEM

Leishmaniasis constitute a group of vector-borne protozoan diseases among which some forms are mutilating and/or life-threatening to man. At present, there are no adequate means to protect military personnel required to operate in endemic areas of these diseases. Immunization of troops prior to deployment could be the best means of conferring protection. The feasibility of this approach is suggested by the long-lasting immunity conferred to individuals with healed cutaneous and/or visceral leishmaniasis. However, the mosaic of antigens in the organism presents a major problem. These antigens must be isolated, purified and characterized, and their capacity to stimulate immunity must be ascertained in order to establish their potential protective value. Also, it is necessary to delineate clearly the roles of cell-mediated and humoral immunity in these diseases. The present study is designed to explore immunity in leishmaniasis and to provide a better understanding of the mechanisms involved.

RESULTS AND DISCUSSION OF RESULTS

The formation of circulating immune complexes in hamsters infected with *Leishmania mexicana* was demonstrated. Anti-complementary activity (AC) in sera collected from normal hamsters and from others infected with *L. mexicana* was assayed. Of the normal sera, 9 of 33 were AC, whereas in sera collected from animals 15 to 17 days, and 60 to 64 days after infection, 4 of 5 and 13 of 39 sera were AC respectively. The incidence of 80% in sera collected 15-17 days after infection suggests that antibody formed early in disease combined with circulating antigen to form immune complexes which rendered the sera AC. There were no apparent differences in AC between hamsters initially infected with either promastigotes or amastigotes.

Sera collected from hamsters infected with *L. braziliensis* and from hamsters infected with *L. mexicana* which did not show AC were tested for complement fixing (CF) and precipitating antibodies. In sera obtained from animals 17 to 64 days following infection with *L. mexicana*, 14 of 80 were CF positive. A greater number of sera (16 of 41) from a comparable group of animals infected with *L. braziliensis* showed CF antibodies. In both groups of sera, CF antibody titers ranged from 1:4 to 1:64. The greatest number of reactors occurred among animals tested 48 to 55 days after infection. None of the sera demonstrated precipitating antibody by immunodiffusion or counter-current immuno-electrophoresis. The failure to detect consistently antibody early in the disease, and the variation in the frequency of reactors point to the transient nature of leishmanial antibody. This is consistent with the hypothesis



that antibody elicited early in disease forms immune complexes in the presence of excess antigen and is not detectable as free antibody.

Several antigenic preparations of L. mexicana were evaluated for their ability to elicit high-titer anti-leishmanial sera in rabbits. These preparations included (1) crude soluble promastigote fraction, (2) soluble fraction with sodium alginate adjuvant, (3) dialyzed soluble fraction, (4) whole promastigotes, (5) whole promastigotes with sodium alginate adjuvant, (6) insoluble pellet, (7) insoluble pellet with sodium alginate adjuvant, (8) insoluble pellet treated with sodium desoxycholate, and (9) insoluble pellet treated with sodium desoxycholate and dialyzed. The data showed that L. mexicana preparations induced the production of 180 50% units of sheep red blood cell hemolysin, CF antibody of up to 1:32 titer, and precipitating antibody of 1:2. In general, CF and hemolytic antibodies were detected 7 days after the first immunizing dose, whereas, precipitating antibodies were not detected until 30 to 51 days after the first immunizing dose. These data show that immunization of rabbits with leishmanial antigens results in the sequential production of antibodies of different specificities. Comparable antigenic preparations of L. braziliensis were injected in rabbits and are currently being evaluated.

The ability of L. mexicana antigens to induce a rise in sheep red blood cell hemolysin is evidence for the presence of Forssman antigen in Leishmania. The source of this antigen has not been ascertained at this time. However, the implications of this finding relative to species differentiation and host resistance are currently being explored.

#### CONCLUSIONS

The high incidence of AC in sera collected from hamsters infected with L. mexicana suggests the formation of immune complexes. Their formation appears to offer a rational explanation for the transient nature of leishmanial antibody and its illusiveness. The consistent weak immune responses of rabbits to various antigenic preparations of L. mexicana antigens indicates that Leishmania antigen homogenates may consist of substances which are inhibitory to antibody induction and production. The finding of Forssman antigen in Leishmania may prove important to species differentiation and host resistance.

#### RECOMMENDATIONS

The presence of circulating immune complexes in American leishmaniasis should be further substantiated by direct evidence and their role in protective immunity should be assessed. The finding of Forssman antigen in Leishmania should be investigated to determine its significance in relation to species differentiation and host resistance. Leishmanial homogenates should be evaluated to identify components which contribute to delay in antibody induction.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)656	
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3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'N INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
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23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Capabilities will be provided to respond to the requirements of the DOD Food RDT&amp;E Program (AR 70-3) for nutrition studies for all military services. Objectives are (a) to provide essential information on the nutritional adequacy of feeding systems, rations, and dietary standards for military personnel in all environs; (b) to evaluate the nutritional intakes and status, physical status, body composition, and work capacity of military to ensure that performance is not impaired by improper nutrition; (c) to evaluate the chemical and nutrient composition of present and future rations; and (d) to provide statistical and computer support for these objectives.</p> <p>24. (U) Information is obtained through clinical examinations, dietary diaries, food analyses and intake measurements, blood and urine biochemistries, anthropometry and physical performance tests. Data are mathematically and statistically evaluated by computer programs. Unique military nutrition problems submitted by the services are studied through workshops, symposia, or research.</p> <p>25. (U) 76 10 - 77 09 An Institute Report has been prepared comparing average nutrient intakes and food wastes before and after implementing the BAS/a la Carte feeding system at Loring AFB. Reports are being finalized and individual's dining hall and total intakes from diaries; blood and urine biochemistries; and processing of dining hall and clinical data have been completed on the NAS-Alameda study of the BAS/a la Carte system. First survey of the Twentynine Palms MCB study of a novel feeding system was conducted. Dietary diaries, clinical and biochemical evaluations, and dining hall surveys were used. Initial reports have been prepared. The before-phase of a modernized ship's feeding system was conducted aboard the aircraft carrier U.S.S. Saratoga CV-60 by dietary diary-interview techniques.</p>							

# ABSTRACT

PROJECT NO. 3M6277A811 Military Nutrition and Food Hygiene  
WORK UNIT NO. 001 Nutrition Studies in Support of DOD Food Program

The following investigations have been conducted under this work unit:

STUDY NO. 3 Nutrition survey at Loring AFB, Maine

STUDY NO. 4 Nutrition survey at Naval Air Station, Alameda, California

ADDENDUM A Phase II of study to evaluate changes in nutrient intakes after renovation of dining facility and initiation of a la carte feeding system

ADDENDUM B Phase III of study to evaluate the biochemical and clinical indices of nutritional status and survey food intakes (in dining hall, other sources, and total) of sailors on the COMRAT a la carte system

STUDY NO. 5 A series of nutrition surveys to evaluate the effects of changing feeding systems (to an all COMRAT/a la carte system) at Twentynine Palms, California, upon nutrient consumption and nutritional status of the Marine

STUDY NO. 6 A before-and-after series of nutrition surveys to evaluate the effects of modifications to Navy food service system afloat on nutrient intake

STUDY NO. 7 Investigation on the effects of initiating a central food preparation facility upon nutrient consumption and wastes in military dining facilities (Fort Lee, Virginia)

STUDY NO. 8 Evaluation of the MCI and MRE rations as the sole subsistence for an extended period

STUDY NO. 9 Nutrient intake data system--nutrient factor file

STUDY NO. 3. A study of the nutritional impact of initiating an all BAS/a la carte item pricing system of feeding enlisted personnel was conducted at Loring AFB, Maine, by monitoring average food intakes and wastes in the dining hall operating under the conventional system and



again after the new system was in operation for several months. Although percent plate wastes were reduced with item pricing, dining hall utilization was also reduced. Customer preference for the short-order type of meal was increased in the new system, but may have been related to the increased variety of items offered in the a la carte system. Fat consumption (over 40% of the calories) was excessive in both systems. Although the average consumptions of nutrients per meal per patron were comparable between the two systems, the reduced utilization of the dining hall operating under the BAS/a la carte system may have reduced the total daily intakes of those personnel who did not have ready access to other dining facilities.

STUDY NO. 4. A further study of initiating the BAS/a la carte item pricing system was conducted at Naval Air Station, Alameda, California. This study consisted of three surveys -- the first and third included 17-day average food consumptions and wastes in the dining facility, and 17-day dietary diary, clinical, biochemical and anthropometric evaluations of individual sailors, including some personnel who were authorized and some who were not authorized subsistence in the military facility. The second survey was a 14-day diary-interview evaluation of the individuals' consumptions to provide an interim report on the nutritional impact of the test system. Dining hall utilization of rations-in-kind personnel decreased from 31.6% in the conventional system to 11.1% at 6 months after initiating all BAS, and 8.4% at 8 months after its initiation. Further data reduction and analyses are ongoing.

STUDY NO. 5. The first survey of the study of improving the military feeding system of the U.S. Marine Corps was conducted at Twentynine Palms MCB during March 1977. The survey consisted of measuring food consumptions in two dining halls, obtaining dietary diary-interview data for total nutrient consumptions during 14 consecutive days for 340 Marines, and clinical/biochemical nutritional evaluations of these personnel. Several inadequacies of both dining hall and total daily nutrient intakes of Marines were identified and these will be considered when planning menus for the novel feeding concept to be tested at this base. The nutritional problems include excessive fat consumption and less than optimum intakes of vitamins, iron, and calcium, and, in some cases, protein.

STUDY NO. 6. The study of nutrient consumptions of Naval personnel afloat was initiated by a 19-day dietary diary-interview data collection for 203 sailors during July-August 1977. Several problems related to the supply of milk and fresh fruits and vegetables were readily observed. Data are currently being prepared for computer processing for individuals' intakes of the various nutrients. Recommendations for improving the nutrition of the sailor at sea will be forwarded to Natick Research and Development Command to be incorporated into the test feeding system afloat. A second survey of the test system is planned for the coming year to evaluate the nutritional impact of this system.

STUDY NO. 7. A protocol was prepared to study the nutritional influences of incorporating a Central Food Management System (central food preparation and satellite dining halls for serving) as the future military feeding system. This system is currently being tested at Fort Lee, Virginia.

STUDY NO. 8. Another protocol was prepared to determine how long personnel could subsist upon present and future combat rations, what the effects of long-term (up to 90 days) subsistence on rations would be, and how to improve or supplement these rations to assure that personnel's health and performance would not be detrimentally affected by sole subsistence on rations. These studies cannot be initiated under present budgetary restrictions.

STUDY NO. 9. A digital computer-based file of information on foods studied in nutrition surveys is maintained with a system of computer programs entitled "Nutrient Intake Data System - Nutrient Factor File" (NIDS-NFF). The file is referred to as the LAIR Nutrient Factor File, and contains data describing food items and defining the concentration of nutrients in the food items. The system allows user maintenance of the data file and the display of the information contained on the file.

## BODY OF REPORT

WORK UNIT NO. 001

Nutrition Studies in Support of DOD  
Food Program

STUDY NO. 3

Nutrition survey at Loring AFB,  
Maine

### PROBLEM

A nutritional evaluation of the basic allowance for subsistence (BAS)/a la carte food service system concept was conducted at Loring AFB, Maine. The previously existing mixed subsistence-in-kind (SIK)/BAS system was evaluated during a 3-day nutrition survey in October 1974, prior to implementation of the all BAS/a la carte system at Loring AFB on 1 January 1975. The BAS/a la carte system was evaluated during a subsequent 5-day survey conducted during November 1975. Dining hall attendance, in-dining-hall nutrient, and food type consumption data of the average patron were obtained along with information regarding the magnitude of plate waste during both surveys. The food trays of all patrons at each meal were photographed to enable subsequent computation of nutrient intakes per meal of individual customers.

### RESULTS AND DISCUSSION OF RESULTS

An Institute Report entitled Nutritional Evaluation of a BAS/a la Carte Food Service System, Loring AFB, Maine, has been drafted, reviewed by the Publications Review Committee, and is currently being revised for final submission for publication. This report encompasses all aspects of the survey, except for the individual food tray data which have not been evaluated pending development of computer programs to reduce data.

### CONCLUSIONS

Conversion to the BAS/a la carte system did not successfully accomplish the goal of attracting more customers to the military dining hall. Implementation of the BAS/a la carte system reduced dining hall attendance at breakfast (19%), noon time (17%), and supper (26%) meals. The item-pricing component of the new system successfully reduced the plate waste (including inedibles such as bone) of milk and milk products, fish and poultry, grain products, beverages, eggs and egg products, legumes and nuts, and tomatoes by 40% or more. A greater variety of foods was offered at the short-order lunch line under the new system, and this expanded menu greatly increased customer preference for the short-order line. Item-pricing decreased citrus juice consumption at midnight and breakfast meals. The BAS/a la carte system increased ascorbic acid and



decreased energy intakes at the short-order line lunch meal. Thiamin intakes per 1000 kcal improved at the main-line dinner meal under the new system, but no improvement was noted for thiamin per 1000 kcal at the short-order lunch meal. Niacin intakes remained lower than desired at the midnight and breakfast meals. The percentage of calories derived from fat sources was excessive (greater than 40%) for all meal periods under both systems.

#### RECOMMENDATIONS

The BAS/a la carte system should not be broadly expanded to other military installations until more comprehensive studies have been conducted and evaluated. Specifically, the impact of the observed reduction in dining hall utilization on the nutritional adequacy of the total daily intakes (both inside and outside dining hall) of individuals could not be determined from this study. It is uncertain whether young military personnel who are given the cash equivalent (BAS) of the food provided to them as subsistence-in-kind will obtain and consume nutritionally adequate and balanced diets. This is of special concern because the military has provided the individual with a limited amount of cash instead of a meal card and with the opportunity to eat elsewhere rather than at a military dining hall.

STUDY NO. 4

Nutrition survey of the Naval Air Station, Alameda, California

ADDENDUM A Phase II of study to evaluate changes in nutrient intakes after renovation of dining facility and initiation of a la carte feeding system

ADDENDUM B Phase III of study to evaluate the biochemical and clinical indices of nutritional status and survey food intakes (in dining hall, other sources, and total) of sailor on the COMRAT a la carte system

#### PROBLEM

The Department of Defense directed all services to test the all COMRAT/cash a la carte feeding concept. The Navy Food Service System Office submitted a requirement under OPNAVINST 3900.26B (AR 70-3) for an evaluation of the Navy feeding systems. Natick Army Research and Development Command (NARADCOM) responded to this requirement, proposed a test of the COMRAT/a la carte system, and requested an evaluation of the effects of these changes upon nutrient consumptions and nutritional status of enlisted personnel. To evaluate these effects upon nutrition, a three-part study was conducted at NAS-Alameda (March 1975, June 1976, and August 1976).

#### RESULTS AND DISCUSSION OF RESULTS

The first survey was conducted to obtain baseline data upon the

conventional feeding system for comparisons of future changes. It included (a) a 17-day dietary diary-interview collection of individual food consumptions from 133 enlisted personnel, (b) clinical and biochemical evaluations of 211 enlisted men, and (c) a 17-day survey of food consumptions and wastes in the dining hall. The second survey was conducted to provide an interim report to the Navy on the changes in nutrient consumptions of sailors after initiating the BAS/a la carte system and was conducted by a 14-day dietary diary-interview collection of data for 154 men. Results of these comparisons were presented in the 1976 Annual Research Progress Report. The third survey was conducted after biochemical and clinical indices had sufficient time to stabilize. It included (a) a 17-day dietary diary-interview collection of individual food intakes of 156 sailors, (b) clinical and biochemical evaluations of 219 men, and (c) a 17-day survey of average food consumptions and wastes in the dining hall. The diary-interview data of the third study confirmed the observations of the second survey, and it was found that utilization of the dining hall by RIK personnel was reduced to 8.4% compared to 11.1% in June; attendance had been 31.6% during the first survey when the conventional system was in effect. Processing and verification of the clinical and biochemical and in-dining hall data have been completed, and statistical comparisons and evaluations will begin immediately.

#### CONCLUSIONS AND RECOMMENDATIONS

1. Conclusions and recommendations of FY 76 Annual Research Progress Report were confirmed.
2. Further conclusions and recommendations will be developed for the evaluations of the data presently being statistically analyzed.

STUDY NO. 5

A series of nutrition surveys to evaluate the effects of changing feeding systems (to an all COMRAT/ a la carte system) at Twentynine Palms, California upon nutrient consumption and nutritional status of the Marine

#### PROBLEM

The effects of changing feeding systems upon the nutrient consumptions and nutritional status of the Marine were evaluated to assure that his/her military capabilities are not adversely affected by poor nutrition. Any change in the feeding system has the potential of influencing the nutritional status of personnel. The Marine Corps submitted a requirement to the DOD Food RDT & Eng Program for an analysis of the food service system at Twentynine Palms MCR. The analysis was to include alternatives for improving this system. Operational Research and Systems Analyses Office (OR/SA) of NARADCOM is conducting a test of a new system (several types of dining facilities, e.g., A ration dining halls,

short-order dining halls, steak houses, and specialty houses serving ethnic foods). The test is a multi-year effort with individual goals of changing decors of dining facilities, updating kitchen equipment and cooking methods, modernizing serving concepts, and evaluating Marine Corps operation of the system after test personnel have relinquished control. A definitive evaluation of the nutritional impact of the new system upon the Marine would require a series of surveys, one of the conventional system for control data, and one after each goal has been attained.

#### RESULTS AND DISCUSSION OF RESULTS

The initial survey to obtain control data from the conventional feeding system was conducted 28 February-20 March 1977, at Twentynine Palms MCB. The survey included individual nutrient consumption data for 14 consecutive days from 340 Marines (including some men and some women); average food consumption during 8 days in a Force Troops dining hall and 7 days in a Student dining hall; and clinical, biochemical, and anthropometric evaluations of the Marines who provided individual dietary information. The results indicated that the average meal consumed in each dining hall contained at least one-third of the Daily Dietary Nutrient Allowances; however, most of the meals contained over 40% of the calories as fat; the concentrations (based on calories) of iron and vitamin A were too low in most meals; concentrations of thiamin, niacin, and ascorbic acid were low in some meals; and total plate wastes averaged 8.8 and 8.6% of the amounts served in the two dining halls.

Individual nutrient consumption (from food, beverages, and nutrient supplements) was collected from five selected groups within the Twentynine Palms population, i.e., three Rations-in-Kind (RIK) and two Commuted Rations (COM) groups. These were composed of (a) males from combat and combat support units (RIK-MC and COM-MC); (b) males from student and headquarters units (RIK-MS and COM-MS); and (c) females from student and headquarters units (RIK-FS). The average daily nutrient intake for all groups is shown in Table 1. Table 2 presents the percentage of each population with average daily nutrient intakes below the military nutritional standards (MDA). Only the RIK-MS group met the energy allowance; between 40-88% of all populations fell below the MDA for iron, vitamin A, and thiamin. Other nutrients of low intake included vitamin C for all groups; calcium for the COM-MC, COM-MS, and RIK-FS groups; and niacin for RIK-MS and RIK-FS groups. All groups ate only about two meals per day; no group ate more than half its meals at the dining hall. On the average, the meals consumed by all groups at the dining hall were nutritionally adequate, although iron intake was low for the females and the proportion of calories from fat slightly exceeded the allowance for all groups.

#### CONCLUSIONS

The average meals consumed in each dining hall surveyed contained at



least one-third of the Daily Dietary Nutrient Allowances; however, most of the meals exceeded the < 40% of calories from fat standard, and the concentrations of iron and vitamins (based on calories) were low in many of the meals. The dietary diary-interview data indicated that Marines consumed 2.1 meals per day, and RIK males consumed 46%, females 24%, and commuted rations personnel < 5% of these in the dining hall. Large percentages of these Marines (up to 93% of the personnel for certain nutrients) consumed less of various nutrients than the military recommended allowances during the 14 consecutive days of study. Even when the average nutrient consumption of a group of Marines was adequate by military standards (and this occurred in < 50% of the cases) large numbers of individuals consumed < 70% of their allowances.

#### RECOMMENDATIONS

1. The fat content of the military menu should be reduced.
2. More food with higher levels of vitamin A, iron, and thiamin should be used in military feeding.
3. It should be investigated whether or not energy needs are being met at the observed energy intake.
4. An educational program should be instituted to increase awareness of their need to increase their total daily intake of protein, vitamin A, iron, thiamin, niacin, and vitamin C. Females also need to increase their calcium and riboflavin intakes.
5. Iron supplements should be made readily available to the female population and they should be encouraged to use them.
6. Personnel should be encouraged to eat in the military dining halls more frequently because these provide nutritionally adequate meals more consistently than meals personnel eat elsewhere.

STUDY NO.

6

A before-and-after series of nutrition surveys to evaluate the effects of modifications to Navy food service systems afloat on nutrient intake

#### PROBLEM

The Department of the Navy requested that a study be conducted aboard a Forrestal class aircraft carrier to define the problems that exist with Navy afloat food service systems. They requested that qualitative and quantitative alternatives to the existing system be developed to achieve improvements in user acceptance, greater efficiency in operations, reduced costs and manpower requirements, and that architectural and design concepts be proposed to improve the total food service environment.

LAIR was tasked with the responsibility of conducting studies to assess the adequacy of the nutritional intakes of Naval personnel in shipboard situations, both prior to and following modifications to the food service system.

#### RESULTS AND DISCUSSION OF RESULTS

A nutrition survey to study the existing Navy afloat food service system was conducted aboard the aircraft carrier USS Saratoga CV-60 during the period 11 July to 8 August 1977. The daily diary-dietary interview technique was used to gather total daily nutrient intake data while the USS Saratoga was in transit from Mayport, Florida, to Rota, Spain, and during the initial two weeks of operational deployment in the Mediterranean Sea. A total of 19 days of food consumption data was obtained from 203 enlisted (E-6 and below) personnel, representing a cross-section of the entire ship's company and attached air wing group. In addition, demographic and anthropometric data and information regarding work hours, temperature in work and berthing areas, and level of physical activity were obtained from each participant.

#### CONCLUSIONS

Initial impressions, prior to reduction and careful evaluation of the data, indicated that the nutritional quality of the food that was offered and readily available to the enlisted sailor was not always adequate to provide him with the optimal amount of nutrients to perform safely his arduous and oftentimes hazardous duties in a stressful environment. A number of problem areas were identified that jeopardize the nutritional health and well-being of sailors at sea. Due to limited refrigerator and freezer storage space, the ship can carry only a 4 to 5 day supply of fresh milk, and resupply occurs only when the ship is in port. The ship was resupplied at sea with fresh fruits and vegetables that had been procured and packaged four weeks earlier. Consequently, all of the lettuce was rotten, and the other fresh produce had greatly deteriorated. Limited availability of refrigerated drinking fountains, and, on occasion, contaminants in the water contributed to excessive consumption of sodas. During prolonged, extensive flight operations and other similar activities, men frequently miss meals because their duty requirements do not allow them the time to stand in line for 15 minutes or longer to get a full course meal. The health and safety of the man, his equipment, and the successful accomplishment of his mission are jeopardized when the man is required to work continuously for 8 to 16 hours without the opportunity to consume a square meal.

#### RECOMMENDATIONS

1. Milk should be made available to the crew at all times. This could be accomplished by more frequent resupply at sea, by increasing refrigerator and freezer space, by the use of ultra-high-temperature sterilized milk, or by providing sufficient can sterilization equipment so that the use of powdered milk could be expanded.

2. Appropriate corrective measures should be taken to assure that the ship is resupplied with fresh fruits and vegetables, and not with produce that has been procured and packaged four weeks earlier.
3. Food Service should use beverage base powder with ascorbic acid added instead of powder without ascorbic acid.
4. The number of refrigerated drinking fountains should be increased to reduce the crew's dependence on cold sodas as the most readily available source of potable drinking fluid.
5. The galleys and mess decks should be modified to speed up the food delivery process and thereby reduce the time a man has to wait in line to get food.
6. There is a definite need to offer a dieter's plate in the galley or post a suggested dieter's menu at the beginning of each serving line.

STUDY NO. 7

Investigations on the effects of initiating a central food preparation facility upon nutrient consumption and wastes in military dining facilities (Fort Lee, Virginia)

#### PROBLEM

A proposal was prepared to evaluate the effects of a new feeding system upon nutrient consumptions and nutritional status of military personnel. Changes in military feeding systems can affect the nutrient intakes and nutritional status of personnel in several ways, including (a) number of meals per week that the person eats in the military dining hall, (b) the nutritional balance of the menu offered by the dining hall, (c) the selection and amounts of foods served to or taken by the patron, (d) the amounts of these foods consumed by the patron, (e) the concentrations of nutrients in the consumed foods (as affected by methods of preparation, storage, and serving of foods), and (f) the amounts and nutritional adequacy of foods consumed from sources other than the military dining facility. A Central Food Management System (CFMS) is being tested by U.S. Troop Support Agency to try to increase economy, efficiency, and utilization of military dining facilities. This system incorporates a centralized food preparation facility (CFPF) and satellite serving facilities. If the concept is successful, the intent is to expand its use throughout the Army, and possibly to other services where it is applicable. A study of the nutritional impact of the test should be conducted prior to its acceptance as the future feeding system.

#### RESULTS AND DISCUSSION OF RESULTS

A protocol has been prepared and submitted to MRDC; however, future



funding guidance precludes initiation of the study. Design of the study was to conduct two dining hall surveys, one before and one after initiating the CFPF concept, including determinations of amounts of foods used by the kitchen, wasted in the kitchen, served to the average patron, and wasted by the patron. Each survey would be for eight days in two dining halls of different sizes and would include data on the age, height, weight, sex, and food habits of the patrons of the dining facilities.

#### CONCLUSIONS AND RECOMMENDATIONS

Investigations are required to assure that the health and military capabilities of the personnel are not adversely affected by improper nutrition before the CFMS is expanded to other posts and services. Many of these nutritional effects require long periods of time to develop and to be detected. Expenditures of funds for incorporating this system at various military bases (these would be larger expenditures!) should be prevented until the system is adequately tested, so as to preclude additional expenditures to correct the system or change the system.

STUDY NO. 8

Evaluation of the MCI and MRE rations as the sole subsistence for an extended period

#### PROBLEM

No long-term study has been conducted on the use of either the present MCI or future MRE combat ration as sole source of subsistence; however, military contingency plans for the future include this concept. Although each meal of these rations contains at least one-third of an adult's allowance of all known required nutrients (excluding water) when procured, both rations are comprised of only 12 meals and long-term stability of some vitamins is not assured. With only 12 meals available (and food preferences of individuals would reduce the number acceptable to him or her), the menu would become monotonous and total nutrient consumptions would be expected to be drastically reduced. Deterioration of labile vitamins could result in nutritional deficiencies in the consumer that would adversely affect his/her mental and/or physical performance. Therefore, it is imperative that this study be conducted before we have a military operation with only rations available. Excessive casualties due to inadequate nutrition may compromise the entire operation.

#### RESULTS AND DISCUSSION OF RESULTS

A draft protocol has been prepared, a coordination meeting was conducted with all commands involved, and MRDC indicated that they would solve the problem by rewriting some regulations to allow long-term use of rations and make local commanders responsible for personnel consuming the entire ration for as long as required.

## CONCLUSIONS AND RECOMMENDATIONS

A long-term feeding study with each ration as the sole source of subsistence should be conducted. Personnel's food consumption, nutritional status, body weight and composition, mental and physical capacities, and military performance of duties should be monitored. This study should be conducted before rations are accepted as the only food available during a military operation.

STUDY NO.            9

Nutrient intake data system - nutrient factor file

## PROBLEM

A nutrition survey includes a detailed monitoring of foodstuffs consumed by individuals and groups, in military dining facilities and away from them, both from observations and from the analysis of diaries kept by individuals. The analyses of these data require the use of a computer due to their large volumes and complexity. To effect these analyses, information is needed about the foods consumed. A file of information on foods, both from handbooks and from laboratory analyses, is needed as well as computer programs for the maintenance of the file.

## RESULTS AND DISCUSSION OF RESULTS

At the U.S. Army Medical Research and Nutrition Laboratory, Denver, Colorado, computer programs were written and used on a second generation digital computer for the maintenance of a file of data on foods patterned after the Department of Agriculture Handbook No. 8. This program served well for the design of a system of programs to maintain a detailed file of information which describes food items and defines the concentration of nutrients contained in the food items. These data are derived from standard handbooks, published literature, and in-house laboratory analyses. The variables of information stored on file, the LAIR NUTRIENT FACTOR FILE, are detailed in Table 3.

The programs which maintain and display the data file are entitled the "Nutrient Intake Data System - Nutrient Factor File" (NIDS-NFF). These programs operate on a Control Data Corporation Model 7600/6600 digital computer at the Lawrence Radiation Laboratories, Berkeley, California. They allow the user to maintain the file through the use of pre-printed keypunch forms so that he can add food items or data on existing food items, change variables, or delete data from the file. The user can print various combinations of data from the file, sorted in a number of ways, for desk use. Classes of foods can be retrieved for use in other computer programs as well as format the entire file in various ways for differing types of programs. For example, data can be formatted from the file for use by programs which were written for the older version of the nutrient factor file while programs are expanded to use the new

file directly. The system has been designed for ease of use by the casual user, thus reducing the reliance of the user on computer personnel.

#### CONCLUSIONS AND RECOMMENDATIONS

1. The NIDS-NFF programs have been placed in routine use.
2. Data on foods collected during nutrition surveys should be filed in an orderly manner on the LAIR Nutrient Factor File.
3. Users should be encouraged to make use of the data contained on the file in their user programs.

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TABLE 1  
AVERAGE DAILY NUTRIENT INTAKE<sup>1</sup>  
TWENTYNINE PALMS MARINE CORPS BASE

NUTRIENT	RIK-MC	COM-MC	RIK-MS	COM-MS	RIK-FM
ENERGY (KCAL)	2795 ± 1365 <sup>2</sup>	2569 ± 1316	3167 ± 1269	2621 ± 1222	1922 ± 1036
PROTEIN (G)	98.2 ± 51.4	101.0 ± 58.5	113.9 ± 50.7	106.1 ± 63.6	69.1 ± 41.9
FAT (G)	111 ± 63	109 ± 66	130 ± 60	115 ± 80	84 ± 52
% FAT CALORIES <sup>3</sup>	35.6 ± 12.1	38.6 ± 31.3	37.2 ± 10.3	38.8 ± 19.0	23.7 ± 12.0
CARBOHYDRATE (G)	284 ± 141	267 ± 152	330 ± 142	257 ± 137	212 ± 124
CRUDE FIBER (G)	3.2 ± 2.3	3.1 ± 2.5	3.5 ± 2.5	3.3 ± 2.9	2.5 ± 2.2
CALCIUM (MG)	1068 ± 773	927 ± 787	1344 ± 779	1018 ± 862	812 ± 729
PHOSPHORUS (MG)	1320 ± 788	1344 ± 805	1460 ± 731	1426 ± 775	914 ± 527
CA:P RATIO <sup>3</sup>	0.9 ± 1.0	0.7 ± 0.5	1.0 ± 0.8	0.7 ± 0.5	1.0 ± 1.2
IRON (MG)	14.3 ± 8.0	17.8 ± 13.3	15.9 ± 8.3	20.1 ± 15.8	32.4 ± 116.0
VITAMIN A (IU)	4433 ± 8087	5237 ± 9635	4991 ± 6903	5387 ± 8805	4434 ± 7047
VITAMIN C (MG)	80 ± 94	87.9 ± 103.2	86 ± 101	131.7 ± 286.2	121 ± 203
THIAMIN (MG)	1.26 ± 0.84	1.73 ± 1.94	1.48 ± 0.92	3.47 ± 14.96	4.60 ± 15.0
(MG/1000 KCAL) <sup>3</sup>	0.47 ± 0.32	0.77 ± 1.91	0.47 ± 0.21	1.65 ± 8.96	2.93 ± 11.01
RIBOFLAVIN (MG)	2.42 ± 1.42	1.94 ± 3.93	2.80 ± 1.49	3.53 ± 6.32	4.39 ± 12.07
(MG/1000 KCAL) <sup>3</sup>	0.59 ± 0.47	1.31 ± 2.71	0.89 ± 0.35	1.60 ± 3.88	3.01 ± 10.44
NIACIN (MG)	23.5 ± 14.0	26.6 ± 21.5	24.4 ± 14.3	31.0 ± 39.4	31.2 ± 73.6
(MG/1000 KCAL) <sup>3</sup>	8.7 ± 4.9	12.6 ± 27.7	7.6 ± 3.3	13.4 ± 23.2	20.5 ± 63.0

<sup>1</sup> AVERAGE DAILY NUTRIENT INTAKE = INTAKE FROM FOODS, BEVERAGES, AND NUTRIENT SUPPLEMENTS.

<sup>2</sup> MEAN ± STANDARD DEVIATION.

<sup>3</sup> MEAN CALCULATED FROM INDIVIDUAL NUTRIENT RATIOS.

TABLE 2  
PERCENTAGE OF POPULATIONS  
WITH AVERAGE DAILY NUTRIENT INTAKES  
BELOW MILITARY NUTRITIONAL STANDARDS<sup>1</sup>  
TWENTYNINE PALMS MARINE CORPS BASE

NUTRIENT	RIK-MC	COM-MC	RIK-MS	COM-MS	RIK-FS
Energy	75.0	75.6	56.1	80.6	64.3
Protein	67.3	53.5	31.6	51.5	71.4
% Fat Calories	73.1	59.3	71.9	55.3	61.3
Calcium	30.8	41.9	17.5	42.7	57.1
Phosphorus	11.5	7.0	1.8	6.8	45.2
Iron	88.5	67.4	82.5	56.3	85.7
Vitamin A	71.2	61.6	59.6	61.2	81.0
Vitamin C	40.4	47.7	33.3	35.9	45.2
Thiamin	78.8	64.0	61.4	62.1	59.5
Thiamin/1000 kcal	78.8	43.0	78.9	40.8	61.9
Riboflavin	32.7	37.2	19.3	39.8	45.2
Riboflavin/1000 kcal	9.6	9.3	10.5	3.9	19.0
Niacin	44.2	37.2	49.1	47.6	54.8
Niacin/1000 kcal	19.2	3.5	36.8	6.8	45.1

<sup>1</sup> Departments of the Army, the Navy, and the Air Force. AR 40-25/  
BUMEDINST 10110.3E/AFR 160-95. Medical Services Nutritional  
Standards. Washington, DC: 30 August 1976.

TABLE 3  
CONTENTS OF LAIR NUTRIENT FACTOR FILE

<u>Descriptive Type Information</u>	
Food item number	- Identifies the food with unique identifying number
Food description	- Describes the food item
Meal code	- A code which allows the investigator to specify a particular meal in a particular survey where this food was collected for analysis
Supplemental food description	- Allows additional detailed description of the food item
Normal serving size	- Aids menu analyses
Food source code	- Defines the type of food, e.g., dairy products, fruits
Food subgroup code	- Allows more detailed definitions of the food group, e.g., citrus fruit, dried fruit
Course code	- Describes the primary use for the item, e.g., entree, dessert
Spare code	- Is unused at the present time to allow expansion of the system
<u>Food Preparation Information</u>	
Refuse	- Indicates the percent of the food which is lost during preparation
Preparation type	- Defines how this particular entry in the file was prepared, e.g., baked, fried. Data are included in this category also for indirectly calculating the nutrient concentration of foodstuffs, as recipes with corrections made for cooking losses, e.g., due to heat and evaporation

(NOTE: All the remaining data in the file are concentrations of nutrients and are stored with a code indicating the source of the data, e.g., Department of Agriculture, LAIR analysis)



Table 3. CONTENT OF LAIR NUTRIENT FACTOR FILE (Cont)

<u>Proximate Analysis</u>		
Water		
Available energy calculated from protein, fat, and available carbohydrate in kcal and kjoules		
Protein		
Total lipids		
Total carbohydrates		
Crude fiber		
Ash		
<u>Minerals</u>		
Calcium	Copper	Nickel
Iron	Manganese	Cadmium
Magnesium	Molybdenum	Arsenic
Phosphorous	Selenium	Mercury
Potassium	Chromium	Lead
Sodium	Vanadium	Fluorine
Zinc	Tin	Four spare fields for expansion of mineral data
Chlorine	Silicon	
Iodine		
<u>Vitamins</u>		
Ascorbic acid	Retinol	Vitamin K
Thiamin	Alpha Carotene	Niacin (total activit
Riboflavin	Beta Carotene	Pyridoxal
Niacin	Gamma Carotene	Pyridoxine
Pantothenic acid	Cryptoxanthin	Pyridoxamine
Vitamin B <sub>6</sub>	Vitamin D	Folic acid (available
Vitamin B <sub>12</sub>	Vitamin E activity	Expansion of vitamin data
Vitamin A in IU and in Retinol equivalents	Alpha Tocopherol	

TABLE 3. CONTENT OF LAIR NUTRIENT FACTOR FILE (Cont)

<u>Amino Acids, Dietary Fiber, and Sucrose</u>		
Tryptophan	Histidine	Proline
Threonine	Arginine	Hydroxyproline
Isoleucine	Cysteine	Serine
Leucine	Cystine	Three spare fields for the expansion of amino acid data
Methionine	Glycine	Dietary fiber
Phenylalanine	Aspartic acid	Sucrose
Valine	Glutamic acid	Total animal protein
	Tyrosine	Total vegetable protein

<u>Fatty Acids</u>		
Total Saturated fatty acids	Polyunsaturated fatty acids	Cholesterol
4:0	18:2	Phytosterols
6:0	18:3	Total animal fat
8:0	18:4	Total vegetable fat
10:0	20:4	Total monounsaturated fatty acids
12:0	20:5	
14:0	22:5	16:1
16:0	22:6	18:1
18:0	Polyunsaturated to saturated ratio	20:1 22:1

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUM. <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DISB'H INSTR' <sup>a</sup>	8b. SPECIFIC DATA- CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM A. WORK UNIT
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
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				NAME: Amos, W., MAJ, MS; Waring, P., DAC			
				NAME: Omaye, S., DAC; Tillotson, J., DAC			
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Military men and women may be placed in stressful situations that can significantly alter their nutritional requirements and influence their military effectiveness and health. One general objective is to conduct research pertaining to the adequacy of military diets and nutrient requirements, intake and nutritional status of all military personnel. The investigations are aimed to provide increased knowledge concerning (a) nutritional requirements, (b) nutrient content of modern military foods, and (c) effects of certain food or food items on metabolism.</p> <p>24. (U) Through use of animal experiments and controlled human studies, we will seek to (a) define the nutrient requirements of military personnel in terms of age, sex, and activity levels; (b) establish the nutritional parameters essential for the military personnel to perform with maximum physical and mental capability under all environmental and military stresses; (c) develop methods for determining nutrients and biochemical components in blood, tissues, urine, and foods for application in military medicine, nutrition surveys, and rations studies; (d) develop techniques which reflect the nutritional status; (e) determine whether or not various stresses, diseases, or injuries increase nutrient requirement.</p> <p>25. (U) 76 10 - 77 09 Various folate radioassay procedures were evaluated with the microbiological assay on human serum and whole blood samples. Radioassay procedures appear satisfactory for use on serum samples but difficulties have been encountered with whole blood samples. Procedures for the analysis of vitamin C in serum and blood samples were also evaluated. A serum ferritin assay procedure has been developed and applied to military surveys. Trace mineral analytical procedures were developed and applied to foods obtained from military nutrition surveys. An improved erythrocyte transaminase procedure has been developed for assessing vitamin B-6 status.</p>							

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.



# ABSTRACT

PROJECT NO. 3M762772811 Military Nutrition and Food Hygiene  
WORK UNIT NO. 002 Nutritional Requirements of Military  
Personnel in Health, Injury and  
Disease

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Requirements, functions, interactions and metabolism of ascorbic acid (vitamin C)
- STUDY NO. 2 Riboflavin - metabolism and analytical methods
- STUDY NO. 4 Evaluation and development of procedures and techniques for the assessment of the trace mineral nutritional status of military personnel
- STUDY NO. 5 Nutritional iron deficiency
- STUDY NO. 7 Techniques to evaluate nutritional status of humans

STUDY NO. 1. Evaluations of three methods of ascorbate analysis demonstrated that only the 2,4-dinitrophenylhydrazine test was satisfactory for the determination of ascorbate in whole blood or red blood cells. Ascorbate depletion and repletion in *Cynomolgus* monkeys had little effect on iron or copper distribution, drug metabolism or the profile of urinary ascorbate metabolites. The uptake of  $^{14}\text{C}$ -ascorbate in platelets and red blood cells was somewhat inversely related to the plasma ascorbate status of the animal. After monkeys ingested  $^{14}\text{C}$ -ascorbate, the feces, (uncontaminated by urine), had measurable amounts of  $^{14}\text{C}$ -compounds. Erythorbate supplementation had little effect on the parameters measured, except to decrease the half-life of antipyrine metabolism.

STUDY NO. 2. Flavoproteins, one specific for riboflavin and one for flavin mononucleotide, have been isolated from egg whites and a micro-organism, respectively. Studies are in progress to establish if fluorometric titrations with purified apoproteins would be a quantitative and sensitive measure of flavins in biological and diet samples.

STUDY NO. 4 Procedures have been developed for the analysis of trace minerals in foods. Foods sampled from the dining facility at the Alameda Naval Air Station were analyzed for copper, zinc, manganese, iron, and magnesium. Dietary intakes of copper, zinc, and manganese were calculated for personnel eating at the above dining hall. Intakes of zinc and manganese appeared to be adequate. However, copper intakes in general were below the 2 mg/day required for balance. The implications of marginal copper intakes are not known at this time.

STUDY NO. 5. Serum ferritin levels were measured in 352 individuals (310 males, 42 females) at the Twentynine Palms Marine Corps Base as an index of iron stores. Females had significantly lower levels of serum ferritin than males. No such difference was found for serum iron, the traditional index of storage iron.

STUDY NO. 7. Four commercially available folate radioassay kits have been tested on serum and whole blood samples from two military nutrition surveys and the results compared with those obtained with the use of the L. casei assay. The serum values were compatible. The Bio-Rad folate radioassay kit is recommended as an acceptable alternative to the microbiological folate assay for serum samples. However, whole blood values differed sufficiently to warrant further studies before relying on the radioassay procedure as the diagnostic tool for red cell folate.

## BODY OF REPORT

WORK UNIT NO. 002

Nutritional Requirements of Military Personnel in Health, Injury and Disease

STUDY NO. 1

Requirements, functions, interactions and metabolism of ascorbic acid (vitamin C)

### PROBLEM

The experiments reported in this study were initiated in order to advance further the fundamental knowledge concerning the nutrition and biochemistry of ascorbic acid. Research is needed to provide improved means to assess the ascorbate adequacy in military personnel. Means for assessment must be sensitive, relatively fast and specific. Unfortunately, basic information on the mechanism and function of ascorbic acid is incomplete and fragmentary; subsequently, it is difficult to recommend intakes of ascorbate to military personnel, especially under adverse or stress situations. Therefore, basic research must be undertaken to provide the understanding of ascorbate's biological function. Research should include the metabolism and nutrient interactions as well as the pharmacology and toxicology of ascorbate. Once some insight into these functions is determined, recommendations of ascorbic acid intakes can be made for optimal health and performance of military personnel.

### RESULTS AND DISCUSSION OF RESULTS

*Cynomolgus* (*M. fascicularis*) monkey(s), fed a modified Lieber and DeCarli diet, demonstrated a direct relationship between the amount of dietary ascorbic acid and the amount of ascorbic acid determined in whole blood, plasma, or red blood cells (RBC). An allowance of 100 kcal per kg of body weight, adequate in ascorbate, seems to be a generous enough food intake in the maintenance of adult monkeys. Three photometric methods of ascorbate analysis were evaluated in monkey plasma, whole blood and RBC. All three methods were comparable for plasma ascorbate; however, neither procedure using  $\text{Fe}^{+3}$ /ferrozine reduction or 2,6-dichloroindophenol (DCIP) reduction proved as satisfactory as 2,4-dinitrophenylhydrazine condensation (DNPH) in the specificity of whole blood or red blood cell ascorbate. In contrast to guinea pig experiments, supplementation of monkeys adequate in ascorbate with isoascorbic acid twenty times the ascorbate level (10 mg/kg) did not depress total blood ascorbate.

Uptake of ascorbate into monkey blood cell components. Direct DNPH measurements of ascorbic acid in plasma, RBC, and whole blood during ascorbate depletion and repletion, indicated that those blood values paralleled the dietary treatment. However, indirect measurements of



buffy coat ascorbate, i.e., the difference between total blood ascorbate and the sum of red blood cell and plasma ascorbate, suggest that this blood fraction retains its ascorbate content to a greater extent than other blood fractions during ascorbate depletion. In general, isolated RBC and platelets in vitro have an inverse relationship between  $^{14}\text{C}$ -ascorbate uptake and ascorbate content. The linear correlation coefficients for plasma ascorbate versus RBC and platelets are  $-0.38$  ( $P < 0.005$ ) and  $-0.19$  ( $P < 0.1$ ) respectively. There appears to be no correlation between plasma ascorbate and white blood cell ascorbate uptake,  $-0.05$  ( $P > 0.1$ ). In contrast to previous reports of guinea pigs and taking into account the degree of data scatter, ascorbate uptake by blood cell components does not seem to be useful in the determination of ascorbate status. In addition, erythorbic acid dietary supplementation had no influence on the uptake of ascorbate in isolated blood cell components.

Metabolism of ascorbate. A single oral dose of  $1\text{-}^{14}\text{C}$ -ascorbate to ascorbate depleted or repleted monkey, resulted in the excretion of urine that presented similar carbon-14 chromatographic profiles. Forty-eight hours after the label was fed, the diethylaminoethyl cellulose urinary ascorbate chromatographic profile was shifted. Urine samples contained at least six labeled metabolites and the neutral fraction, containing any free ascorbate, contained less than 20% of the label. Non-specificity of ascorbate analysis was illustrated when two metabolites could be detected by the  $\text{Fe}^{+3}$ /ferrozine reduction and five were detected by the DNPH method. Contrary to previous studies, the feces, uncontaminated by urine, contained measurable labeled material throughout the 10 days the feces were collected.

Copper and iron. Copper exists normally as ceruloplasmin in plasma (80% of plasma copper). Ascorbate depletion and repletion had no general effect on monkey ceruloplasmin. Likewise, ascorbate depletion and repletion had no effect on iron metabolism as determined by red cell count, hemoglobin, hematocrit, mean corpuscular volume, reticulocyte count, and serum iron. In addition, repletion of monkeys with ascorbic acid and supplemented with high levels of erythorbic acid had no effect on copper or iron parameters.

Drug metabolism and body temperature. No significant correlations between levels of ascorbic acid in plasma and sleeping or body temperature were observed during any phase. Further, no significant correlation between body weight and sleeping time was found. These results are consonant with experimental results and conclusions found in the scientific literature regarding the influence of ascorbic acid on body temperature. Although no influence by ascorbic acid on ketamine sleeping time was found, the duration of the sleeping time is influenced not only by metabolism, but also by redistribution factors in the body. The influence of ascorbic acid on body distribution factors was not assessed in this experiment; but the experimental results suggest bio-distribution to be the major factor effecting ketamine sleeping time.

Contrary to experiments in guinea pigs, ascorbate depletion and repletion did not alter antipyrine distribution. However, the additional supplementation of erythorbic acid did reduce the half-life of antipyrine to 50% of that found when the monkey was in an ascorbate depleted state. In contrast, theophylline metabolism was not altered regardless of dietary treatment. Although more work is needed, the findings indicated above suggest that the requirement for vitamin C for drug metabolism in monkeys is different than the requirement in the guinea pig and that the site of action of ascorbate may be directed more toward P-450 oxidation than P-448 where theophylline is acted upon.

### CONCLUSIONS

In many respects the role of vitamin C in monkeys differs from that demonstrated in the guinea pig. These differences include: the influence of ascorbate on uptake of labeled ascorbate in blood cell components, on drug metabolism, and on ascorbate metabolism. Because of its degree of sensitivity and specificity, only the DNPH method of ascorbate analysis proved useful in assessing the whole blood or RBC ascorbate status in the depleted state. Ascorbate status did not influence plasma copper or iron levels; however, this finding is not unusual since the mineral content of the diet was adequate.

### RECOMMENDATIONS

1. Specific methods that are sensitive and fast for the direct measurement of body fluid ascorbate must be developed. High pressure liquid chromatography (HPLC) appears to be an approach worthy of investigation.
2. Experiments need to be designed to understand further and to define the difference in ascorbate utilization-metabolism in the monkey versus the guinea pig, and to determine how useful the monkey model can be extrapolated to man.
3. The mechanism of action of ascorbate and isoascorbic acid in monkey hepatic drug metabolism should be clarified.
4. The influence of ascorbic acid on marginal intakes of copper and/or iron needs to be determined.
5. Urinary and fecal ascorbate metabolites should be isolated and identified.
6. The role of ascorbic acid in body temperature control in thermally stressed animals must be determined.

STUDY NO. 2

Riboflavin - metabolism and analytical methods

#### PROBLEMS

During military surveys the riboflavin nutritional status of personnel is measured by enzymatic or microbiological assay; however, it is difficult to measure the severity of the deficiency. The microbiological assay for riboflavin in biological and diet samples is time consuming and can be non-specific. A quick, sensitive and direct measure of riboflavin and flavin nucleotides would be desirable. Recently, two flavoproteins have been isolated, one which is specific to riboflavin and the other to flavin mononucleotide. The fluorescence of the flavins is quenched when the apoprotein combines with an equimolar concentration of its flavin. Few compounds exhibit fluorescence similar to the flavins; therefore, a fluorometric titration of biological or diet extracts using known amounts of the apoenzyme should provide a quantitative measure of riboflavin and flavin mononucleotide. The flavin dinucleotide is enzymatically hydrolyzed to the flavin mononucleotide for measurement.

#### RESULTS AND DISCUSSION OF RESULTS

The egg white apoprotein specific for riboflavin has been purified and initial studies suggest that fluorometric titration with the apoprotein quantitatively measures urinary riboflavin. The microorganism containing the flavin mononucleotide specific apoprotein was obtained after considerable delay from an investigator in Germany. Purification of this protein is being studied. Progress on the study has been curtailed by the loss of a technician during the first quarter of the year.

#### CONCLUSION

The egg white apoprotein has been purified and will be used to develop a procedure to measure urinary riboflavin quantitatively.

#### RECOMMENDATIONS

Investigations to purify the flavin mononucleotide specific apoprotein should be continued. It should be determined if the two flavin apoproteins can be used as the basis for quantitative fluorometric titration assays of riboflavin in biological and diet extracts. These procedures should be compared with the microbiological riboflavin assay.

STUDY NO. 4

Evaluation and development of procedures and techniques for the assessment of the trace mineral nutritional status of military personnel



## PROBLEM

Little is known about the trace element requirements of man or current dietary intakes of these elements. Rapid changes in food resources and food processing portend alterations in the levels, ratios, and availability of essential mineral elements in military feeding systems. Thus, it is important to know the requirements and levels of essential trace minerals in foods consumed by the military in order to assure optimum performance, health and recovery from injury.

## RESULTS AND DISCUSSION OF RESULTS

A procedure has been developed for the analysis of various trace minerals in foods sampled from military feeding systems. The food samples were homogenized to milkshake consistency by adding a measured amount of water as necessary. The samples were then freeze dried. Because of the lack of an adequate hood system, samples were ashed in a low temperature ashers where excited oxygen radicals were passed over the samples. The ash was then dissolved in 10 ml of 0.05 N  $\text{HNO}_3$  and the mineral elements determined by atomic absorption spectrometry. With the use of this procedure, excellent agreement was obtained in the analyses of the National Bureau of Standards of bovine liver and orchard leaf standards for copper, zinc, manganese, and magnesium.

Over 250 different food items, sampled from the serving line at the mess hall at the Alameda Naval Air Station have been analyzed for copper, zinc, manganese, magnesium, and iron by the above procedure. Where comparison could be made, there was reasonable agreement with literature values. These values are currently being placed in the LAIR nutrient factor file.

Intakes of copper, zinc, and manganese have been calculated for personnel attending the Alameda Naval Air Station cafeteria during the initial survey of March 1975. Data obtained under Work Unit 001 relating to average dining hall intakes and dietary recall records of selected individuals were combined with the above data for these calculations.

Average zinc intakes of about 20 mg/person/day and manganese intakes of 2.5 mg/person/day compared favorably with estimated requirements of 15 mg/day for zinc and 2.5 mg/day for manganese. However, about 70% of the individual daily intakes of manganese were below 2.5 mg/day. Average copper intakes of about 1.7 mg per day were significantly below the 2.0 mg per day considered necessary for balance. It was estimated that 75% of the individual daily intakes were below 2.0 mg/person/day. Copper is essential for collagen and elastin synthesis, combating infections and many oxidase type enzymes. Deficiencies in any of the above may impair recovery from injury and could impair optimal performance.

## CONCLUSIONS

It is not known at this time the full significance of the marginal copper intakes found in the above study.

## RECOMMENDATIONS

1. The data base for trace mineral contents of foods should be expanded to cover more food items and other essential and toxic minerals.
2. Other military populations need to be surveyed as to their trace mineral intakes in order to determine if potential problems exist relating to trace element nutriture.
3. The effects of marginal or low intakes of various essential trace minerals on performance and recovery from injury should be more thoroughly investigated.
4. Human requirements for the essential trace minerals need to be more carefully defined, particularly under various stress situations.

STUDY NO.            5                                    Nutritional iron deficiency

## PROBLEM

Results from several nutrition surveys of military installations indicate that approximately 3% of males and 14% of females have serum iron levels below acceptable limits. However, serum iron measurements may not adequately detect lowered stores of iron in the body. Recently, serum ferritin has been shown to be a better index of iron stores than serum iron. Such a measurement is necessary in the military setting since there is evidence that iron deficiency without accompanying anemia may lead to impairment of muscle function.

The objective of this study was to assess the iron storage of military populations by means of ferritin analysis and to study those factors in the diet which influence iron absorption and metabolism.

## RESULTS AND DISCUSSION OF RESULTS

A commercial kit (Ferr-Iron, Ramco, Inc.) was evaluated and found suitable for the purpose of measuring ferritin in human serum. Serums from the Twentynine Palms Marine Corps Base nutrition survey were analyzed. There were 312 males and 42 females in the subject population. Ferritin data along with other parameters of iron status are presented in Table 1.

TABLE I

VARIOUS IRON STATUS INDICATORS

	<u>MALES</u>	<u>FEMALES</u>	<u>SIGNIFICANCE</u>
Hemoglobin (g/dl)	17.1 $\pm$ 1.1 <sup>1</sup>	14.9 $\pm$ 1.2 <sup>2</sup>	<.01
Hematocrit	48.1 $\pm$ 2.6 <sup>1</sup>	41.0 $\pm$ 2.6 <sup>2</sup>	<.01
Mean Corpuscular Hemoglobin Content	35.2 $\pm$ 1.6 <sup>1</sup>	35.7 $\pm$ 1.4 <sup>2</sup>	<.05
Serum Iron (ug/dl)	107 $\pm$ 36 <sup>3</sup>	103 $\pm$ 45 <sup>2</sup>	NS
Total Iron Binding Capacity (ug/dl))	352 $\pm$ 39 <sup>3</sup>	395 $\pm$ 63 <sup>2</sup>	<.01
% Total Iron Binding Capacity Saturation	30.7 $\pm$ 10.7 <sup>3</sup>	26.5 $\pm$ 11.0 <sup>2</sup>	<.01
Serum Ferritin (ng/dl)	62 <sup>5</sup> (3-87) <sup>4,6</sup>	16 <sup>5</sup> (14-266) <sup>2,6</sup>	<.01

<sup>1</sup>N=312, <sup>2</sup>N = 42, <sup>3</sup>N=311, <sup>4</sup>N = 310, <sup>5</sup>Geometric mean, <sup>6</sup>95 percentile range

Preliminary examination of the data indicates that the female population has a significantly lower serum ferritin level than the male population (16 ng/ml vs 62 ng/ml). No such difference was seen for serum iron in the subject population. The value of 16 ng/ml for females is borderline low for the female population and may indicate a risk for female troops with respect to iron stores.

While males have significantly greater levels of hemoglobin and higher hematocrit values, the female population has a slightly, but significantly higher mean corpuscular hemoglobin level, which may indicate an adaptive measure to insure adequate oxygen supply to the tissues.

CONCLUSIONS

Since analysis of the data is incomplete at this time, firm conclusions cannot be drawn. However, it is already apparent that serum ferritin levels more readily detect marginal iron status.

RECOMMENDATIONS

Further work needs to be performed to establish the incidence of lowered iron stores in military populations and to assess the ability of this parameter to indicate the severity of the nutritional deficiency.



Lowered iron stores, especially in the case of dietary deprivation of meat protein, may result in some individuals being unable to perform at required levels under the stress of battle situations.

Further analysis of the data, especially correlations of serum ferritin levels with dietary iron intake, may indicate whether the lower ferritin levels are due to low iron intake or represent a lowered iron absorption rate. While females, especially at the age of most women in military service, and young males have generally lower indices of iron status than the adult male population, little is known about the effect of such a deficiency on work performance. The first step in assessing this problem is to establish the extent of nutritional iron deficiency in military populations.

STUDY NO. 7

Techniques to evaluate nutritional status of humans

#### PROBLEM

The purpose of this study was to evaluate and improve current methods for assessing folic acid nutritional status. Microbiological assays for serum and red cell folate have been used traditionally, but recently several commercial folate radioassay kits have become available. Although they are becoming widely employed, their diagnostic reliability, particularly with respect to red cell folate, has not been established. The purpose of the first experiment was to compare several kits with the L. casei method which has already been established as a diagnostic tool.

#### RESULTS AND DISCUSSION OF RESULTS

Four commercially available folate radioassay kits (Bio-Rad, New England Nuclear (NEN), RIA Products, and Amersham) have been tested on whole blood and plasma samples from Alameda III and Twentynine Palms military nutrition surveys. The data from each assay have been compared with those obtained from the L. casei "reference" method by statistical procedures, including scatter plots, regression lines, correlation coefficients, and analysis of variance.

The radioassay procedures offer the advantages of speed, technical ease and the fact that they are less affected by antibiotics or other drugs which may be present in human blood samples. In addition, these assays respond to the conjugated vitamin as well as to the monoglutamate, in contrast to the L. casei method which requires 1 to 2 h incubations to release red cell folate.

Serum Analyses. All kits except NEN yielded results compatible with the L. casei assay. The NEN kit values were approximately 25% low and did not correlate well with the L. casei method ( $r = 0.52$ ). The Bio-Rad

kit was found to give the best agreement with the microbiological assay; the means were not statistically different and  $r = 0.86$ . The RIA Products kit yielded reasonable results, but was found less acceptable for technical reasons. On the basis of a limited number of serum samples, the Amersham kit appeared to be acceptable. The latter two kits were both designed for serum analyses and were therefore not tested on whole blood samples.

Whole Blood Analyses. The values obtained with both the NEN and Bio-Rad kit differed significantly from those of the L. casei assay (and, from each other). The NEN kit was again found to be unacceptable. Better agreement was obtained with the Bio-Rad kit, although there were many discrepancies. Both the mean and the range of 95% of values were significantly higher by the Bio-Rad assay compared with the L. casei values. If the lower limits for "normal" and "marginal" values are slightly adjusted upward for the Bio-Rad assay to compensate for the higher average values, there still remain discrepancies as to whether individual samples are "deficient" or not. In order to assess the relative diagnostic potential of the two assays, it will be necessary to compare them with samples from animals (and humans) as they are made progressively folate deficient under controlled conditions.

#### CONCLUSIONS

The radioassay procedures (in particular, the Bio-Rad kit) were found to agree well with the L. casei assay for serum folate. However, discrepancies were found in the whole blood analyses and thus the diagnostic capability of the kits cannot be assumed for red cell folate. Although the radioassay procedures are potentially useful for red cell folate analyses, further studies are needed on samples from subjects with known folate status and under controlled conditions of dietary folate intake.

#### RECOMMENDATIONS

Because of technical advantages and a greater sample handling capacity, the Bio-Rad folate radioassay kit is recommended as an acceptable alternative to the microbiological assay for future serum folate analyses at this Institute. However, further studies are needed to establish the reliability and diagnostic value of the kit for the analysis of whole blood folate.

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11. SAUBERLICH, H.E., and M.L. BROWN (Editors). Proceedings of workshop on human vitamin B<sub>6</sub> requirements. National Academy of Sciences (In press)
12. TILLOTSON, J.A., and A.A. SOMERA. A sensitive assay of tissue ascorbate using Fe<sup>+++</sup>/Ferrozine reagent. Manuscript submitted to LAIR Publication Review Committee for publication



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6337	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62172A	3M162172A811		00	003		
B. SECONDARY	62772A	3M762772A811		00	003		
C. TERTIARY	CARDS 114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Analytical Biochemistry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry; 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER: Not Applicable				FISCAL YEAR		77	
C. TYPE:				CURRENT		4.0	
D. KIND OF AWARD:				78		116	
E. AMOUNT:				4.0			
F. CUM. AMT.							
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Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Sauberlich, H.E., DAC			
				NAME:			
				POC:DA			
22. KEYWORDS (Precede Each with Security Classification Code)							
(U) Analytical Biochemistry; (U) Instrumentation; (U) Automated Analyses; (U) Nutrition Surveys; (U) Clinical Chemistry; (U) Mil Medicine							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objectives are to develop and adapt new concepts in analytical biochemistry to provide reliable and advanced procedures and services to military-oriented research programs at LAIR and, on occasion, to approved cooperating agencies; to innovate or develop analytical procedures to meet specific needs of such research as, for example, the development of micro-automated assay procedures for enzymes related or altered during nutritional deficiencies, disease states, or stress conditions; to develop procedures applicable to military nutrition surveys, ration test studies, and food wholesomeness evaluations.</p> <p>24. (U) Analytical support will be provided to studies in military medicine requiring routine analyses in volume or unique equipment and special techniques for assays of physiological specimens obtained during military nutrition surveys or field studies. Specific analyses will be originated or adapted, as required, to meet the needs of specific studies and to improve the economy and efficiency of laboratory operations. Research will be conducted on a continuing basis in support of the objectives indicated to provide new methods. Whenever feasible and practical, the methods will be automated and linked to computer systems.</p> <p>25. (U) 76 10 - 77 09 Analytical support requiring approximately 28,000 analyses was provided to 46 research projects from 6 departments at LAIR. A procedure for determining serum ascorbic acid was developed for use on a centrifugal fast analyser. Inter-method evaluations were made on procedures for serum calcium, magnesium, and ascorbic acid.</p>							

# **ABSTRACT**

PROJECT NO. 3M762772A811 Military Nutrition and Food Hygiene  
WORK UNIT NO. 003 Analytical Biochemistry

The following investigations have been conducted under this work unit:

STUDY NO. 1 Analytical support and services

STUDY NO. 2 Development of analytical biochemistry procedures

STUDY NO. 1. Analytical support requiring 31,395 individual analyses was provided to 46 research projects.

STUDY NO. 2. A method for determining serum ascorbic acid employing dye reduction was adapted to the centrifugal analyzer. Significant improvements were implemented in the amino acid and centrifugal analyzer systems.

## BODY OF REPORT

WORK UNIT NO. 003

Analytical Biochemistry

STUDY NO. 1

Analytical support and services

### PROBLEM

Chemical analysis of various physiological specimens and diet or ration items is a fundamental adjunct to the majority of the research objectives of the many research protocols active at LAIR. The Analytical Biochemistry Branch has the responsibilities for providing service to the investigating staff in the form of automated analyses and special chemistries.

### RESULTS AND DISCUSSION OF RESULTS

The Branch provided support to 46 research projects, including two field studies, which resulted in a total of 31,395 automated, semi-automated, and manual analyses. These analyses were distributed by type as follows:

<u>Analytical Service</u>	<u>Number of Analyses</u>
<b>Blood Chemistry:</b>	
(1) Automated electrolytes, glucose, total protein, urea nitrogen, creatinine, iron, cholesterol, triglycerides, vitamin C, lactate, pyruvate, uric acid, hemoglobin, and various enzymes	21,845
(2) Semi-automated lipid phosphorus and total iron binding capacity	789
(3) Manual catecholamines	627
<b>Urine Chemistry:</b>	
(1) Automated electrolytes, creatinine, urea nitrogen, and hydroxyproline	5,375
(2) Semi-automated methylmalonic acid	502
(3) Manual catecholamines and xanthurenic acid	846
<b>Food and Tissue Chemistry:</b>	
(1) Semi-automated minerals	55
(2) Manual proximate analyses	624
<b>Special Chemistry:</b>	
Amino Acids	732



These analyses were distributed among the Departments as follows: Surgery, 37.4%; Nutrition, 27.8%; Medicine, 17.5%; Comparative Medicine, 13.2%; Biomedical Stress, 3.8%; and Dermatology, 0.3%. The two field studies (Work Unit 001, Nutrition Studies in Support of DOD Food Program) required 7,041 analyses.

#### CONCLUSIONS

Although branch staffing was reduced to 80% of the previous level at the beginning of the fiscal year, it managed to service promptly all requests for analyses which were or could be automated. The automated analysis load increased 99% over the previous year.

The output of semi-automated and manual analyses decreased to about 70% of the level of last year. A considerable backlog has developed because the deficiencies mentioned last year still exist (i.e., lack of perchloric acid hood and required hooding of solvent extraction systems) and another hood had to be removed from service in March due to unresolved engineering problems in the central exhaust system. The backlog of samples requires 14,787 analyses, most of which must be carried out in hooded areas of either type. It is estimated that these analyses would require 450 man-days of effort.

The overall analytical output increased 69% over last year.

#### RECOMMENDATIONS

Knowledge of the concentration of various constituents of physiological and diet specimens is essential to the conduct of mission-oriented research programs of the Institute. It is recommended that the Analytical Biochemistry Branch continue to function as the central resource for such support. Elimination of the cited deficiencies and reduction of the backlog should be prime goals; however, the additional loss of two technician positions as the fiscal year closed presents a serious obstacle to progress.

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STUDY NO.

2

Development of analytical biochemistry  
procedures

PROBLEM

Automation or simplification of analytical procedures is required to expand analytical support capabilities and maintain operational efficiency. Improvement of method quality is a continuous concern and innovation of procedures is occasionally required. Staff must be prepared to advise investigators on analytical approaches.

RESULTS AND DISCUSSION OF RESULTS

The automated amino acid analyzer was retrofit with hardware and software improvements providing greater dynamic range, improved peak area counting, simultaneous 440 and 590 nm baseline monitoring and post analysis normalization on the basis of an internal standard.

The centrifugal analyzer was retrofit with hardware and software improvements providing improved thermal control in the cuvette housing enabling rapid temperature adjustment and early absorbance readings with zero-time extrapolation to enable background blanking adjustments.

The dye reduction method for ascorbic acid was adapted to the centrifugal analyzer. Values obtained on the same samples by this and the continuous flow method have been collected for statistical evaluation.

Values for calcium and magnesium determined by a photometric centrifugal analyzer method, a photometric continuous flow method and atomic absorption on the same samples have been collected for statistical evaluation.

CONCLUSIONS

The equipment retrofit additions have significantly improved the performance and operational efficiency of the system involved.

Intermethod comparisons are needed to determine if substitutions in methods can be made to meet contingencies such as system failure and sample size restrictions.

RECOMMENDATIONS

Continuous monitoring of operations with the objectives of automating, and improving efficiency and quality of performance is required.

PUBLICATIONS

SAUBERLICH, H., W.C. GOAD, J.H. SKALA, AND P.P. WARING. Procedure for mechanized (continuous-flow) measurement of serum ascorbic acid (vitamin C). *Sel Meth Clin Chem* (In press)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DESGN INSTN <sup>a</sup>	8b. SPECIFIC DATA- CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM <sup>a</sup>
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<del>3. TERTIARY</del>	CARDS 114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Military Food Hygiene							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
006500 Food, 007800 Hyg. & Sanitation, 016800 Toxicology, 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
70 04		CONT		DA		C. In-House	
17. CONTRACT/GRANT				19. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
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5. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		6.0	
6. TYPE:				CURRENT		208	
7. KIND OF AWARD:				78		5.0	
8. CUM. AMT.						236	
18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Taylor, Stephen L., DAC			
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				POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Wholesomeness; (U) Data Collection; (U) Microbiological Limits; (U) Safety; (U) Foodborne Disease; (U) Food Contaminants; (U) Methodology							
23. (U) The objectives are to monitor the microbiological quality of food items through laboratory analysis; to review the literature for trends; to report findings; to determine toxicological quality of foods; to establish suggested guidelines for items of potential health hazard; to review wholesomeness and safety requirements for DoD procured subsistence and to make recommendations to OTSG; to evaluate and develop new and existing methodology and equipment in the areas of food microbiology, virology and toxicology; and to collect microbiological data for food items analyzed by DoD laboratories.							
24. (U) The study designed to assess analysts' accuracy in counting agar plates will include a comparison of analysts' counts and automatic colony counting (ACC) machines' counts with a true count. Present findings indicate peroxides are responsible, in part, for the lethal effects of freezing on <u>Clostridium perfringens</u> ; consequently, antioxidants will be included with the cryoprotective chemicals under study. Feeding studies to determine the toxicity of products resulting from insect infestation will be limited to acute short-term studies.							
25. (U) 76 10-77 09 The study of the microbial flora of fresh and frozen ground pork will continue with variations in methodology in an attempt to improve isolation precision both quantitatively and qualitatively. A procedure has been established that will detect less than one plaque forming unit (PFU) of enterovirus per gram of food. In a survey, enterovirus was not detected in 400 food samples. The data collection portion of the study to determine the specific type of <u>C. perfringens</u> isolated from foods has been completed. The survey of the histamine content of selected seafoods and fermented products will continue. Studies are being initiated to assess the efficiency of available methods for the identification of enterotoxin-producing <u>Escherichia coli</u> in foods, and efforts will be made to improve these methods.							

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.



# ABSTRACT

PROJECT NO. 3M762772A811 Military Nutrition and Food Hygiene

WORK UNIT NO. 004 Military Food Hygiene

The following investigations have been conducted under this work unit:

- STUDY NO. 11 Investigations of microbiological methodology performed in conjunction with the Chapter Chairman, agar plate method, for development of the 14th Edition of Standard Methods for the Examination of Dairy Products
- EX-5 A comparison of analyst and automated colony counters
- STUDY NO. 12 Investigations into the microbiological quality of food items purchased by DoD for the purpose of establishing tentative microbiological guidelines for military subsistence
- EX-6 A survey of the microbial flora of soya protein extended ground beef and its components
- EX-7 A survey of the microbial flora of comminuted pork and a comparative study of microbiological and analytical techniques
- STUDY NO. 13 Investigations into the use of group D streptococci as indicators of the hygienic quality of frozen food products
- STUDY NO. 14 Typing *Clostridium perfringens* isolated from foods
- STUDY NO. 15 The effect of cryoprotective agents in the survival of *Clostridium perfringens* type A vegetative cells in selected meat products after freezing and thawing
- STUDY NO. 17 Investigations into potential toxicological problems associated with food items purchased by DoD: Identification of hazardous substances, evaluation of food safety, alleviation of toxicological problems, and establishment of tentative toxicological guidelines for military subsistence

- EX-1 Histamine production by food spoilage micro-organisms and development of an analytical method for detection of histamine in food products
- EX-2 The toxicity of histamine and other biogenic amines as related to food poisoning, the presence of inhibitors of histamine-metabolizing enzymes in foods, dietary conditions, and other gastrointestinal factors
- EX-3 A survey of commercially packed scombroid fish products, luncheon meats, cheese, and sauerkraut for histamine content
- STUDY NO. 18 Investigations into methodology for detecting and identifying viruses in selected Department of Defense subsistence items
- STUDY NO. 19 Investigations into potential insect induced toxicological problems in foods
  - EX-1 Insect toxic products in military subsistence - induction and quantitation
  - EX-2 Benzoquinones from the secretory glands of *Tribolium confusum* and *Tribolium castaneum* - Assay, reactions, effects, and acute toxicity
- STUDY NO. 21 Enterotoxigenic *Escherichia coli* from foods
  - EX-1 Assessment of methods for the identification of enterotoxin-producing *Escherichia coli* strains
  - EX-2 Methodology for isolation of EEC from foods

STUDY NO. 11, EX-5. The manpower requirements for microbiological analysis of food products represents an ever-increasing element of cost. One time-consuming task is the manual counting of agar plates. The use of automatic colony counters (ACCs) in place of manually counting agar plates will result in a significant manpower savings. The ACCs must be evaluated to determine if they provide suitably accurate counts before they can be used in place of manual counts.

STUDY NO. 12, EX-6. Studies have shown that the use of textured soy protein extended ground beef can result in considerable economic savings. The military services have expressed interest in its use as a substitute for raw ground beef. However, the addition of soy protein to ground beef may create a product with altered microbial considerations. In this study, ground beef, textured soy protein and soy protein extended ground beef were analyzed and microbiological characterization was accomplished.

STUDY NO. 12, EX-7. Analyses of 757 samples of varying pork products have been conducted to provide data for the establishment of microbial guidelines for these products. Analyses have been conducted by more than one technique in order that specific analytical methodology for these products be developed. The microbial population of 100 of these samples has been completely characterized.

STUDY NO. 13. Regulatory food microbiologists commonly interpret the presence of *E. coli* as evidence of fecal contamination. Most microbiologic criteria for food products are based on the presence or absence of *E. coli*. It has been shown that the injury suffered by *E. coli* cells when frozen and thawed is manifested by their inability to grow on the selective media used for their enumeration. It has been shown, during this study, that while the group D streptococci and *E. coli* both survive freezing, the streptococci do not suffer injury and are completely recoverable on selective media while *E. coli* cannot be adequately enumerated in this manner.

STUDY NO. 14. Strains of *Clostridium perfringens* originally obtained from foods, were isolated in pure cultures. These organisms were typed by using the serum neutralization technique in mice. Of the 339 strains typed 320 were found to be type A, the only type of *C. perfringens* that has been associated with foodborne illness outbreaks in the United States. The other 19 strains produced insufficient exotoxin for typing.

STUDY NO. 15. *Clostridium perfringens* is probably responsible for a disproportionately high number of the unconfirmed outbreaks of foodborne illness because of its fragility to freezing. Several chemicals have been screened for cryoprotective properties. Those showing the most cryoprotective activity are glycerol, methanol, dimethyl sulfide, propylene glycol, and ethylene glycol. The effect of the addition of catalase to destroy peroxides will also be investigated.

STUDY NO. 17, EX-1. An improved analytical method for the detection of histamine in foods was developed. Several systems and spray reagents were evaluated for use in a rapid thin-layer chromatographic screening method for histamine in foods. Several bacterial species were identified as capable of producing large amounts of histamine in liquid media including *Proteus morganii* and *Enterobacter aerogenes*. Investigations are continuing to assess histamine production in tuna fish broths and to determine optimal conditions for microbial histamine formation.

STUDY NO. 17, EX-2. The mechanism of oral histamine toxicity is perplexing considering the presence of three enzymes in the gastrointestinal tract which can metabolize histamine. Assays for these enzymes were developed and the subcellular localization in rats, guinea pigs, and monkeys was determined. Investigations into the possible existence of foodborne inhibitors of these enzymes and the effect of dietary and gastrointestinal factors in these enzymes is continuing.



STUDY NO. 17, EX-3. Histamine is known to occur in scombroid fish products, certain luncheon meats, cheeses, and sauerkraut. Surveys of the histamine contents of these products, which are currently underway, will aid in assessment of any potential hazards associated with these foods and in development of guidelines for military subsistence, if any are needed.

STUDY NO. 18. A procedure was established for the recovery of virus from foods. The procedure will detect one or more plaque forming units of virus per gram of food sample. Virus was not detected in 406 samples tested: ground beef (116), soy protein (31), pork sausage (108), fresh clams (22), frozen oysters (31), fresh crab muscle (54), and crab viscera (44).

STUDY NO. 19, EX-1. An in-house resource of coleopterous stored-products pests of military subsistence was established for the first time at this Institute for the support of studies on potential insect induced toxicological problems in food. This capability has been expanded to include eleven species reared in selected military subsistence items. Methods were developed for extraction and quantitative analysis of 2-methyl- and 2-ethyl-1,4-benzoquinone and 1-pentadecene in flour beetles. The concentrations of these compounds were determined in two species of *Tribolium* with respect to age and sex.

STUDY NO. 19, EX-2. The toxicity of the benzoquinones released by flour beetles needs to be established. Investigations were begun into the reactions between benzoquinones and amino acids and proteins. These reactions may modify the toxicity of benzoquinones. A knowledge of the kinetics of these reactions, the nature of the reaction products, and the amount of benzoquinones produced by the flour beetles will allow assessment of the amount of unreacted, potentially toxic, benzoquinone, which may remain in the flour following infestation.

STUDY NO. 21, EX-1, EX-2. Recently recognized has been the potential to cause acute gastroenteritis by bacterial species other than *Salmonella* and *Shigella*. Some *E. coli* strains have demonstrated the capabilities for elaboration of enterotoxins involved in gastrointestinal illness. This study intends to assess methods for the selective isolation and identification of enterotoxigenic *E. coli* and to examine military subsistence to determine the incidence of such strains in these products.

## BODY OF REPORT

WORK UNIT NO. 004

Military Food Hygiene

STUDY NO. 11     Investigations of microbiological methodology performed in conjunction with the Chapter Chairman, agar plate method, for development of the 14th Edition of Standard Methods for the Examination of Dairy Products

EX-5             A comparison of analyst and automated colony counters

### PROBLEM

The Department of Defense (DoD) procures many subsistence items in which the procurement specifications prescribe microbiological limits. Many of these specifications refer to Standard Methods for the Examination of Dairy Products (SMEDP) for official methods. SMEDP prescribes the standard methods agar (SMA) plates will be counted when estimating the microbial population of foods. Also prescribed are standards of accuracy, i.e., SMEDP states "laboratory workers who cannot duplicate their own counts on the same plate within 5% and the counts of other analysts within 10% should discover the cause(s) and correct such disagreements." Recent studies under this Work Unit, in which innovative techniques were used to determine the true number of colonies per plate, have demonstrated that SMEDP accuracy standards are far more severe than had been previously recognized. In addition, the counting of SMA plates is monotonous, time-consuming, and is also an unpopular task among analysts.

A number of scientific equipment manufacturers have developed automated colony counters (ACCs) which are claimed to be equivalent or superior to analyst accuracy and result in a considerable manpower savings. In a limited evaluation, ACCs have been found to warrant further investigation both from the speed and accuracy standpoints. An evaluation of ACCs for accuracy and speed is essential prior to their being declared acceptable laboratory instruments for use in the procurement of DoD subsistence.

### RESULTS AND DISCUSSION OF RESULTS

The data collection phase of the study is in progress. The initial data suggest that the ACCs present a significant savings of manpower, and provide acceptable counts on SMA plates of "good quality." A full analysis of the data will be necessary to judge their full potential.

### CONCLUSIONS AND RECOMMENDATIONS

No conclusions will be formulated until all data are collected and analyzed.

## PUBLICATIONS

None

STUDY NO. 12     Investigations into the microbiological quality of food items purchased by DoD for the purpose of establishing tentative microbiological guidelines for military subsistence

EX-6             A survey of the microbial flora of soya protein extended ground beef and its components

## PROBLEM

Soy protein extended ground beef has been used in the civilian community since early 1971 and it is currently used in the Federal School Lunch Program. Nutritionally, this product is similar to ground beef. Economically, it has been shown that 20% soy extended ground beef can reduce ground beef costs by 21% on a raw basis and as much as 30% on a cooked basis. Interest has been shown in using this product in the United States Armed Forces from an economic standpoint.

With the addition of soy protein to raw ground beef a new product has been created. Also a product with new microbiological considerations may result from the addition of soy protein to a raw comminuted product. If this product is to be used by the military services an increased understanding of the bacterial flora is in order. This study quantitated and characterized the microbial flora of soy protein, ground beef, and soy protein extended ground beef both initially and after an additional 7 days storage at 4 C.

## RESULTS AND DISCUSSION OF RESULTS

In this study ground beef (GB), textured soy protein (TSP), and TSP extended ground beef (SGB) were analyzed after 3- and 10-days' storage at 4 C. Analyses included aerobic plate count (APC), psychrotrophic plate count (PPC), coliform most probable number (MPN) and plate determinations (CMPN and CPC), *Escherichia coli* MPN and plate determinations (EHPN and EPC), *Staphylococcus aureus* MPN (SMPN), fecal streptococci count (FSC), *Clostridium perfringens* determinations, isolation and identification of gram-positive and gram-negative organisms and screening for enteric viruses. Statistical analyses of the enumeration procedures showed significant increases in the total microbial flora after 10-days' storage. PPCs were significantly higher than APCs. CMPNs were significantly higher than CPCs for GB and SGB. The EMPNs were significantly higher than EPC in SGB only. *E. coli* was the predominant gram-negative isolate from GB and SGB. Few gram-negatives were found in TSP. *C. perfringens* was the predominant gram-positive isolate in GB and SGB while *Bacillus* sp. predominated in TSP. *Salmonella enteritidis* ser. worthington



was isolated from GB and TSP. No enteric viruses were detected in this study.

#### CONCLUSIONS AND RECOMMENDATIONS

1. The addition of textured soy protein appears to have no effect on the total microbial load of regular GB.
2. If properly handled, SGB is microbiologically no more or less hazardous than GB.
3. Additional studies to evaluate the effect of TSP on specific foodborne pathogenic organisms are warranted.
4. Evaluation of currently accepted incubation times and temperatures for meat analysis is needed.

#### PUBLICATIONS

FOSTER, J.F., J.L. FOWLER, J.T. FRUIN, L.S. GUTHERTZ, E.L. SHROYER, and M.R. SHALABY. A survey of the microbial flora of ground beef, textured soy protein and textured soy protein extended ground beef after 3 days' and 10 days' storage at 4 C. Submitted to LAIR Publications Review Committee as a LAIR Report.

FRUIN, J.T., J.F. FOSTER, and L.S. GUTHERTZ. Comparison of recovery methods for *Clostridium perfringens* from food. Abstracts of the Annual Meeting, American Society for Microbiology, 1977

FRUIN, J.T., J.F. FOSTER, and L.S. GUTHERTZ. Comparison of recovery methods for *Clostridium perfringens* from selected foods. J Food Quality, In Press

EX-7      A survey of the microbial flora of comminuted pork and a comparative study of microbiological analytical techniques

#### PROBLEM

There are several different types of fresh and frozen comminuted pork products which enjoy high volume usage by DoD. Although some microbiological studies have been conducted on these items, literature reports do not contain data that are either sufficient in volume or comprehensive enough to be used for the establishment of microbial guidelines. To date 757 samples have been processed to generate data on the microbiology of the different comminuted pork products for the eventual establishment of microbiological guidelines. In addition, each analysis for coliform organisms, *Escherichia coli*, *Clostridium perfringens*, *Salmonella* sp., and *Bacillus cereus* has been conducted by two procedures in order to determine the optimum

analytical methodology for comminuted pork. Since comminuted pork does have the potential for being a vehicle for the transmission of foodborne pathogens, 100 of these samples have had their microbial population completely characterized.

#### RESULTS AND DISCUSSION OF RESULTS

None; data collection is still in progress.

#### CONCLUSIONS AND RECOMMENDATIONS

None

#### PUBLICATIONS

None

STUDY NO. 13     Investigations into the use of group D streptococci as indicators of the hygienic quality of frozen food products

#### PROBLEM

Methodology currently used for part of the microbiological examination and determination of the hygienic quality of frozen foods is based upon detection and enumeration of *Escherichia coli*. It has been demonstrated that many of the treatments to which foods are subjected may result in sublethal injury to the bacterial cells which may be contained therein. One of the manifestations of sublethal injury is the inability of cells to grow and form colonies on the selective media routinely used for these enumerations. *E. coli* is an organism which has been shown to suffer severe injury following freezing and frozen storage.

This study was undertaken to investigate the use of group D streptococci as indicators of the hygienic quality of frozen food products as well as to develop methodology for rapid speciation within the group.

#### RESULTS AND DISCUSSION OF RESULTS

*E. coli* and the seven species making up the group D streptococci have been examined for their abilities to survive freezing in water, 0.2 M phosphate buffer pH 7.0, 10% sucrose, 5% glycerol and a 15% slurry of mashed potatoes when stored at temperatures of -4, -20 and -80 C.

With *E. coli*, following 180 days of frozen storage at -4 or -20 C in a food product like mashed potatoes, 94% of the original population could be recovered on a nonselective medium. Following storage

in the same menstruum at -80 C, 99% of the original population was recoverable. However, if these cell suspensions were plated on a selective medium, as would be done during routine food analysis, only 63% of the population surviving -80 C storage, 12% of those surviving -20 C storage and none of the population surviving -4 C storage could be detected. A similar situation was found with *Streptococcus bovis* and *Streptococcus equinus* which comprise the non-enterococcal portion of the group D streptococci.

Results with the enterococcal portion of the group D streptococci indicate that this portion of the group survives freezing at all temperatures studied and is nearly completely recoverable on selective media. Of this group, *S. faecalis* and *S. liquefaciens* show the most promise for use as hygienic indicators in frozen products.

The ability for identification of these organisms was developed. With the use of a replica plating device, 32 separate isolates can be inoculated onto each agar plate in the media battery. Gas chromatography procedures for use in speciating these organisms are currently being studied.

#### CONCLUSIONS

Because of its inability to grow on selective media following freezing, *E. coli* should not be used as a hygienic indicator in frozen products. *S. faecalis* and *S. liquefaciens* should be seriously considered for use as hygienic indicators in frozen products.

#### RECOMMENDATIONS

It is recommended that microbial guidelines be developed for frozen products with either *S. faecalis* or *S. liquefaciens* as the indicator organism.

#### PUBLICATIONS

GUTHERTZ, L.S., R.L. OKOLUK, S.L. TAYLOR, and J.L. FOWLER. Hygienic indicator organisms: A comparison of survival and enumeration of group D streptococci and *Escherichia coli* following freezing and frozen storage. Report No. 52. Presidio of San Francisco, CA: Letterman Army Institute of Research, In Press.

STUDY NO. 14    Typing *Clostridium perfringens* isolated from foods

#### PROBLEM

*Clostridium perfringens* can be separated into 5 types (A through E) based on exotoxin produced in culture filtrates. All types are pathogenic to man, animals, or both. Type A is the only type to be considered a major causative agent in outbreaks of foodborne disease. *C. perfringens* type C is known to cause a necrotizing gastroenteritis.



However, this condition appears to be restricted to individuals who are being maintained on a marginal diet which is also low in protein. Type C foodborne disease results when there is a subsequent change to a diet much higher in protein and caloric content. Reports of this disease have been limited to the immediate post World War II in Germany and to tribal feasts in New Guinea. Only type A foodborne disease outbreaks have been reported in the United States. Over the last 4 years there have been 49 confirmed outbreaks and 3679 confirmed cases reported to the Center for Disease Control.

*C. perfringens* is a ubiquitous microorganism and can be routinely isolated from soil, air, water, the intestinal contents of hemothermic animals and from a wide variety of food items particularly from meat items and those raw vegetables commonly contaminated from soil. Since accepted laboratory isolation procedures for *C. perfringens* from foods do not include provisions to determine the type of organism, strains may be isolated that are types B through E, which are not capable of causing foodborne illness. Thus, if a high percentage of *C. perfringens* isolated from food items are of a type other than type A, additional laboratory procedures are necessary to determine if a food has the potential to cause foodborne illness. This study was undertaken to determine the *C. perfringens* type most frequently isolated from foods.

#### RESULTS AND DISCUSSION OF RESULTS

The results of typing 339 strains of *C. perfringens* isolated from six different food items by using the mouse serum neutralization procedure are shown in the table. Isolates of *C. perfringens* that failed to produce sufficient exotoxin for typing on two trials were considered to be non-typeable. Strains of *C. perfringens* isolated from ground beef, ground pork, ground turkey, live crab, cured sausage, and live clams were predominantly type A.

TABLE: Type A isolates of *Clostridium perfringens* from selected foods

<u>Isolate Source</u>	<u>Number of isolates typed</u>	<u>Number of type A isolates</u>	<u>Number of non-typeable isolates</u>	<u>Percent isolates type A</u>
Ground Beef	152	149	3	98
Ground Pork	82	76	6	93
Ground Turkey	59	56	3	95
Live Crab	29	25	4	86
Cured Sausage	13	10	3	77
Live Clams	4	4	0	100
TOTAL	339	320	19	94

A higher proportion of non-typeable strains was found in cured sausage than in either ground beef or ground pork. The higher percentages of non-typeable strains isolated from cured sausage were great enough to warrant speculation as to the cause of the discrepancy, particularly in light of the fact that the components of this product are ground beef and ground pork. The natural fermentation process may favor the survival and growth of *C. perfringens* strains that are low in exotoxin production. The overall incidence of *C. perfringens* isolated from cured sausage by this laboratory is considerably lower than the incidence found in either ground beef or ground pork.

Due to the effort and the expense required in typing strains of *C. perfringens* isolates from these foods when incriminated in outbreaks of foodborne disease and the prevalence of type A *C. perfringens* typing appears to be unwarranted. This is particularly true if epidemiologic information is indicative of *C. perfringens* foodborne disease. However, type A *C. perfringens* may not be predominantly associated with other food items. For example, lamb and veal products which were not involved in this survey are derived from source animals which are commonly associated with *C. perfringens* types B, C and D.

#### PUBLICATIONS

FRUIN, J.T. Types of *Clostridium perfringens* isolated from selected food: A Research Note. Submitted for publication.

STUDY NO. 15    The effect of cryoprotective agents in the survival of *Clostridium perfringens* type A vegetative cells in selected meat products after freezing and thawing

#### PROBLEM

*Clostridium perfringens* type A was characterized as a microorganism capable of causing foodborne illness 24 years ago. Since that time, this organism has become recognized as a major source of foodborne illness in the United States. The reasons for the high incidence of foodborne illness caused by *C. perfringens* are the organism is ubiquitous; it forms spores having high heat resistance; grows well at high temperatures, i.e., 46-50 C; and has a short generation time. The use of bulk quantities in mass feeding facilities provides the conditions favorable for massive outbreaks of *C. perfringens* foodborne disease, particularly if foods are not held at the recommended temperatures of over 60 C.

Historically the causative agent involved in foodborne disease is identified in only about 50% of the outbreaks. Of the large number of unconfirmed outbreaks, it is probable that a disproportionately large share are caused by *C. perfringens* due to the unique cultural

characteristics presented by this anaerobic microorganism. *C. perfringens* is susceptible to destruction at low temperature, particularly freezing, the recommended method of preserving samples suspected of causing foodborne illness when submitted to diagnostic laboratories. Its requirement for anaerobiosis also presents problems of isolation by diagnostic laboratories. As a result the collection, handling, shipment, storage and laboratory preparation of samples has a detrimental effect on the recovery of *C. perfringens*. Cryoprotective agents and agents that reduce the oxidation-reduction potential of food samples, could result in a more precise qualitative isolation of *C. perfringens*.

#### RESULTS AND DISCUSSION OF RESULTS

Actively growing cultures of *C. perfringens* type A vegetative cells were frozen in laboratory preparations of cooked meat with various chemicals added. The chemicals showing the most cryoprotective properties were glycerol, methanol, dimethyl sulfoxide, propylene glycol, and ethylene glycol. Survival of cells was highly variable from one replicate to the next. A number of chemicals tested resulted in decreased cell survival. Recently published data indicate that catalase added to culture medium dramatically increases the recovery of *C. perfringens*. Peroxides that are formed in frozen foods may cause lethal effects; however, if catalase were added to the medium these chemicals would be destroyed. Further testing will be conducted to determine if catalase has a cryoprotective effect.

#### CONCLUSIONS

Glycerol, methanol, dimethyl sulfoxide, propylene glycol, and ethylene glycol are effective to a degree as cryoprotective agents.

#### RECOMMENDATIONS

None

#### PUBLICATIONS

FRUIN, J.T., and F.J. BABEL. Changes in the population of *Clostridium perfringens* type A in a meat medium. J Food Protect 40:622, 1977

STUDY NO. 17     Investigations into potential toxicological problems associated with food items purchased by DoD: Identification of hazardous substances, evaluation of food safety, alleviation of toxicological problems and establishment of tentative toxicological guidelines for military subsistence



EX-1      Histamine production by food spoilage microorganisms  
and development of an analytical method for detection  
of histamine in food products

PROBLEM

Tuna fish, other scombroid fish products, cheese, and sauerkraut which contain abnormally high amounts of histamine have been implicated in several foodborne disease outbreaks. Routine analysis of these foods is necessary but the methods are cumbersome and tedious. The use of sensitive and specific fluorometric detection methods for histamine will be explored. Trials of various extraction methods for selective removal of histamine from food samples will be performed. The use of chromatographic procedures will be evaluated for their potential in removing further interfering substances.

Histamine is formed in foods by microbial decarboxylation of histidine. A large number of microbial species have been reported to possess the requisite enzyme, histidine decarboxylase. However, few quantitative comparisons of the histamine-producing capacities of various microorganisms have been done. A quantitative comparison of over 100 microbial strains on synthetic and tuna fish broth media is planned. Optimal conditions for histamine production will be determined for several of the more prodigious histamine-producing strains.

RESULTS AND DISCUSSION OF RESULTS

Evaluation of several fluorometric and spectrophotometric amine detection methods showed that the fluorometric assay based on o-phthalaldehyde was the most sensitive and specific for histamine detection. The lower limit of detection with this method was 0.05 nmoles of histamine. At equimolar concentrations, interference was detected with histidine and several histidyl dipeptides out of 55 amines tested. At higher concentrations, substantial interference was also found with glutathione, spermidine, cysteine, glycylglycine, carnosine, norepinephrine, and glucosamine.

These interfering substances could be removed by a selective extraction step by using n-butanol to extract the histamine from an aqueous alkaline solution saturated with sodium carbonate. An analytical method for histamine in foods was developed by coupling this selective extraction procedure to the specific fluorometric detection method. The analysis requires sample homogenization in methanol, heating, centrifuging or filtering, extraction, and fluorometric detection of histamine with o-phthalaldehyde. The method can be used to detect histamine in foods that contain as little as 0.02 mg histamine/100 g food. Samples of 20 foods were analyzed including seafoods, comminuted meats, luncheon meats, cheeses, and sauerkraut. Sauerkraut and tuna fish had the highest histamine content among tested foods.

Although the developed method is much less tedious than previous analytical procedures, it still does not lend itself to the rapid screening of large numbers of samples. Therefore, thin-layer chromatography was evaluated as a rapid screening technique. Twelve solvent systems were tested for their ability to separate histamine and histidine on a variety of thin-layer coatings. The best solvent-adsorbent systems were chloroform/methanol/ammonia (2:2:1), methanol/ammonia (20:1), acetone/ammonia (95:5), and double development with (a) n-butanol/acetone/water (2:2:1) and (b) chloroform/methanol/ammonia (12:7:1), all on silica gel layers. Successful separation of histamine from other amine components of methanolic tuna fish extracts was achieved with all four systems. Four different reagents were evaluated for their potential in the detection of histamine on thin-layer chromatograms. Ninhydrin, which gave colored spots, and o-diacetylbenzene, which gave fluorescent spots, seemed to be best, although two other fluorometric detection reagents, o-phthalaldehyde and fluorecamine could also be used. The best solvent systems were methanol/ammonia (20:1) and chloroform/methanol/ammonia (2:2:1) with ninhydrin and these two systems plus acetone/ammonia (95:5) with o-diacetylbenzene. These systems are rapid and selective enough to be used as routine screening methods.

Over 100 different microbial strains were tested for their ability to produce histamine on trypticase soy broth-histidine (TSBH) media and tuna fish broth media. *Proteus morganii*, *Enterobacter aerogenes*, and *Hafnia alvei* showed a quantitative superiority in terms of histamine production when compared to the other tested organisms. Many microbial species known to possess histidine decarboxylase such as *Escherichia coli* produced little histamine suggesting the importance of histaminase in the accumulation of histamine in food products. Some *Enterobacter cloacae* and *Proteus inconstans* strains, *Citrobacter diversus*, and *Proteus rettgeri* formed histamine on TSBH media only.

#### CONCLUSIONS

The developed method for histamine analysis in foods which utilizes a selective extraction step and a specific detection step has advantages over other reported methods in terms of specificity, sensitivity, and accuracy. The new method is less tedious than previous methods. The thin-layer chromatographic screening methods should be useful in the handling of large numbers of samples.

Preliminary evidence suggests that relatively few microbial species have the capability to produce large amounts of histamine in food products. Studies on tuna fish broth media and optimal conditions for histamine formation are continuing.

## RECOMMENDATIONS

None; this study has not been completed.

## PUBLICATIONS

TAYLOR, S.L., and E.R. LIEBER. Specificity and sensitivity of seven histamine detection methods. J Food Sci, 1977, In Press

TAYLOR, S.L., E.R. LIBER, and M. LEATHERWOOD. A simplified method for histamine analysis of foods. J Food Sci, 1978, In Press

LIEBER, E.R., and S.L. TAYLOR. Thin layer chromatographic screening methods for histamine in tuna fish. Manuscript submitted for publication

TAYLOR, S.L., L.S. GUTHERTZ, M. LEATHERWOOD, F.J. TILLMAN, and E.R. LIEBER. Aerobic bacterial histamine production. Manuscript submitted for publication

EX-2      The toxicity of histamine and other biogenic amines as related to food poisoning, the presence of inhibitors of histamine-metabolizing enzymes in foods, dietary conditions, and other gastrointestinal factors

## PROBLEM

Foodborne disease outbreaks have resulted from consumption of foods that contain abnormally high levels of histamine, tyramine, or other biogenic amines. However, histamine can be orally administered to rats in large doses with no apparent development of clinical symptoms of toxicity. The cells of the gastrointestinal tract normally contain three enzymes, monoamine oxidase, diamine oxidase, and histamine-N-methyltransferase, which are capable of metabolizing histamine to nontoxic products. When histamine is ingested with a food sample, some defect must occur in this detoxification mechanism. With tyramine, it is known that toxicity occurs when the tyramine-containing food is consumed in conjunction with a potent monoamine oxidase inhibitor such as tranlylcypromine. No such factors have been associated with food poisoning due to histamine. The possible presence of naturally-occurring or additive foodborne inhibitors of the three histamine-metabolizing enzymes requires investigation. A survey of potential inhibitors which are known to occur in foods or are used as food additives will be conducted.

Dietary conditions might also precipitate histamine toxicity. Diamine oxidase levels are dependent on dietary pyridoxal and riboflavin levels, while histamine-N-methyltransferase levels require adequate amounts of folate and vitamin B<sub>12</sub> in the diet. The effects of dietary deficiencies of these four factors on intestinal activities of the



particular enzymes will be assessed.  $^{14}\text{C}$ -Histamine absorption as a function of dietary levels of these factors will be tested with ligated gut segments. Finally, the in vivo effects of these dietary factors will be determined by observing changes in stomach acid secretion volume and acidity following intraduodenal injection of histamine.

Other gastrointestinal factors, such as histaminase activity in intestinal bacteria, will be investigated also.

#### RESULTS AND DISCUSSION OF RESULTS

Preliminary work has led to development of sensitive radiometric assays for diamine oxidase (DAO) and histamine-N-methyltransferase (HNMT). A spectrophotometric assay was used for monoamine oxidase (MAO). DAO and HNMT activity was found to be localized in the soluble fraction of jejunal mucosa from rats, guinea pigs, and Rhesus monkeys. MAO activity was below detectable limits in rat jejunal homogenates. Work is proceeding on MAO activities in the various subcellular fractions. The effect of potential inhibitors on the activities of these three enzymes will be tested by using the most active subcellular fractions.

#### CONCLUSIONS, RECOMMENDATIONS, AND PUBLICATIONS

None; this study has not been completed.

EX-3            A survey of commercially packed scombroid fish products, luncheon meats, cheese, and sauerkraut for histamine content

#### PROBLEM

An initial survey of 20 food groups (EX-1) revealed that scombroid fish products, certain luncheon meats, cheese, and sauerkraut had the highest histamine contents. Since histamine in foods is potentially toxic, a further survey of the levels in these particular food groups was indicated. Such information can be used to assess the relative risk of histamine poisoning from such foods, to set guidelines for military subsistence where necessary, and to determine the incidence of potentially toxic levels of histamine in foods. Past foodborne disease outbreaks would indicate that histamine levels above 100 mg/100 g should be considered definitely harmful. The surveys will be performed with 30 to 100 samples of each food item.

#### RESULTS AND DISCUSSION OF RESULTS

A survey of fifty samples of sauerkraut revealed an average histamine content of 5.06 mg/100 g. The histamine content ranged from 0.91 mg/100 g to 13.0 mg/100 g among the 50 samples. Such histamine levels are considerably lower than the level of 100 mg/100 g which has been associated with outbreaks of food poisoning.

In a survey of luncheon meat-sausages, it was determined that cooked and semi-dry sausages had much lower histamine contents than fermented, dry sausages. For cooked sausages the mean histamine levels for 30 samples were bologna - 0.55 mg/100 g, cooked salami - 0.83 mg/100 g, and kosher salami - 0.50 mg/100 g. For semi-dry sausages, which often have lactic acid cultures added during short fermentations, the average histamine levels for 30 samples were beef summer sausage - 1.07 mg/100 g, thuringer-cervelat - 2.35 mg/100 g, and thuringer - 1.19 mg/100 g. Dry sausages, which are subjected to natural fermentations for longer time periods, exhibited a higher and more variable histamine content. Three brands each of Italian dry salami and pepperoni were tested with the following mean histamine levels: Italian dry salami (Brand A - 24.5 mg/100 g, Brand B - 2.14 mg/100 g, Brand C - 19.1 mg/100 g) and pepperoni (Brand A - 1.03 mg/100 g, Brand B - 1.42 mg/100 g, Brand C - 38.1 mg/100 g). Chorizo, an uncooked sausage, had an average histamine content of 2.29 mg/100 g for 30 samples. The highest histamine level found in any sample was 55.0 mg/100 g in a Brand C pepperoni. While these levels of histamine are below the 100 mg/100 g level, the brand specific variation indicates that careful control of fermentation would alleviate any problems associated with histamine accumulation. Unwanted growth by bacteria such as *Proteus morganii* is probably the cause of the high histamine levels in certain products.

Surveys of scombroid fish products, including tuna fish, albacore, and mackerel, and cheeses are currently underway.

#### CONCLUSIONS

Sauerkraut is a low risk product for histamine poisoning. Some brands of dry fermented sausages have high histamine content which approach levels that may cause symptoms of toxicity. Italian dry salami and pepperoni might be considered high risk products. Other types of sausages including bologna, cooked salami, kosher salami, beef summer sausage, thuringer-cervelat, thuringer, and chorizo should be considered low risk products in terms of histamine poisoning.

#### RECOMMENDATIONS

None; this study has not been completed.

#### PUBLICATIONS

TAYLOR, S.L., M. LEATHERWOOD, and E.R. LIEBER. Histamine in sauerkraut. Manuscript submitted to LAIR Publications Review Committee

TAYLOR, S.L., M. LEATHERWOOD, and E.R. LIEBER. A survey of histamine levels in sausages. Manuscript submitted to LAIR Publications Review Committee

STUDY NO. 18     Investigations into methodology for detecting and identifying viruses in selected Department of Defense subsistence items

#### PROBLEM

The role of food as a vehicle for virus transmission to humans has not been clearly defined. It is possible that viruses contribute a significant part of the outbreaks of foodborne disease of unknown etiology since laboratory methodology for detection and identification of viruses in foods are not well established. Most of the reports of foodborne viral disease outbreaks are based on epidemiological evidence only and are limited to those diseases with a distinct clinical syndrome, i.e., hepatitis and paralytic poliomyelitis. There have been only a few reports of recovery of viruses found in food items except those found in seafoods. Now, there are a few preliminary reports on the methodology for detecting enteroviruses in foods. These studies involve the laboratory contamination of food items and efforts to recover virus.

The purpose of this study is to establish methods for isolation and identification of viruses from food items and to perform viral surveys of a limited number of foods of military importance.

#### RESULTS AND DISCUSSION OF RESULTS

A procedure was established for the recovery of virus from food samples. Food samples were suspended in tissue culture medium, the suspensions were clarified by centrifugation and filtration through celite and finally, the clarified suspensions were concentrated 40 to 80-fold by molecular filtration. The efficiency of virus recovery was determined by adding a known quantity of virus to a food sample and then measuring the quantity of virus (plaque forming units [PFU]) present following processing.

The average sensitivity of poliovirus type I (POL-I) recovery from 10 laboratory contaminated food samples was 2.6 PFU per gram of food sample. In one experiment known amounts of POL-I virus were added to 5 samples of ground beef (25 g each). The percent of virus recovery ranged from 27 to 76%. The sensitivity of virus recovery was similar for 5 levels of virus added (Table I). Similar results were obtained when  $4.68 \times 10^5$  PFU of POL-I virus was added to 4 different types of food samples (ground beef, pork sausage, crab muscle, and oysters) and one sample of crab viscera (Table II). Again the sensitivity of virus recovery was similar for these 5 samples whereas the percent of virus recovery varied from 40 to 91%. The reduced sensitivity in oysters (5.0 PFU/g) was a direct result of the smaller sample size of 9.5 g (Table II). Based on the data presented in Tables I and II, it appears that the sensitivity as well as the efficiency of virus recovery was independent of the amount of virus present.



TABLE I - Poliovirus type I recovery from laboratory contaminated ground beef

Sample No.	Total Virus Input (PFU)	Virus Detected (PFU/0.1 ml)	Volume of Concentrate (ml)	Virus Recovery (%)	Sensitivity (PFU/g)
1	$2.7 \times 10^7$	$5.7 \times 10^5$	1.3	27	1.9
2	$3.4 \times 10^5$	$4.3 \times 10^3$	3.4	42	3.2
3	$3.4 \times 10^2$	$5.0 \times 10^0$	3.2	47	2.7
4	$6.8 \times 10^1$	$2.0 \times 10^0$	2.6	76	1.4
5	$3.4 \times 10^1$	$0.5 \times 10^0$	2.2	32	2.7
			MEAN	45	2.4

TABLE II - Poliovirus type I recovery from laboratory contaminated food samples\*

Sample	Weight (g)	Virus Detected (PFU/0.1 ml $\times 10^2$ )	Volume of Concentrate (ml)	Virus Recovery (%)	Sensitivity (PFU/g)
Ground beef	22	96.5	2.2	45	2.2
Sausage	24	100.0	1.9	40	1.9
Crab muscle	17	128.5	3.3	91	2.1
Crab viscera	18	99.0	3.5	74	2.6
Oysters	9.5	98.0	2.6	54	5.0

\* $4.68 \times 10^5$  PFU of poliovirus type I was added to each sample

The data in Tables I and II are based on the average number of PFU detected in 2 cell cultures, each inoculated with 0.1 ml. The inoculation of 0.5 ml per cell culture resulted in 2.6 times more plaques. When the total number of PFU for 2 cell cultures, each inoculated with 0.5 ml is used for the calculation of sensitivity, the procedure will detect < 1 PFU/g of food sample.

Virus was not detected in 406 food samples by a procedure that will detect one or more PFU/g of food sample (Table III).

TABLE III - Samples tested for food-borne virus

<u>Food Item</u>	<u>No. of Samples Tested</u>	<u>Host Cell Culture</u>
Ground beef	116	Vero, BT-8
Soy protein	31	Vero, BT-8
Pork sausage	108	Vero, PK-15
Clam	22	Vero
Oyster	31	Vero
Crab muscle	54	Vero
Crab viscera	<u>44</u>	Vero
TOTAL	406	

#### CONCLUSIONS

The procedure established in this study is suitable for the testing of a variety of food items for viral contamination. The absence of detectable virus in these food items is compatible with the lack of reports on the isolation of virus from foods and a recent (1977) report of negative findings for virus in samples from 7 food processing plants and 60 retail food samples.

#### RECOMMENDATIONS

This procedure should be employed for the testing of food items where foodborne viral disease occurs or is suspected.

#### PUBLICATIONS

FOSTER, J.F., J.L. FOWLER, J.T. FRUIN, L.S. GUTHERTZ, E.L. SHROYER, and M.R. SHALABY. A survey of the microbial flora of ground beef, textured soy protein and textured soy protein extended ground beef after 3 days and 10 days storage at 4 C. Submitted to LAIR Publications Review Committee as a LAIR Report

SHROYER, E.L. Food as a vehicle for virus transmission. (Abstract) JAVMA. In Press

STUDY NO. 19 Investigations into potential insect induced toxicological problems in food

EX-1 Insect toxic products in military subsistence - induction and quantitation

## PROBLEM

The military, with its necessity for transport and extended storage of foods in environments favorable to insect infestation, is concerned about the potential health hazards associated with chemicals released into military subsistence by insects. Several species have been recognized as important pests of military subsistence and many of these stored-product insects are known to release chemicals into infested food products. The nature and toxicity of many of the chemicals remains to be determined. At present no satisfactory methods are available for the detection of insect-induced chemicals in the contaminated subsistence. Toxicological evaluation of these chemicals is impossible in the absence of standardized methods for induction and quantitation of the suspected material. The present study is designed to establish an in-house resource of selected insects of military subsistence, to develop the required methodology, and to investigate conditions which promote the release of toxic chemicals.

## RESULTS AND DISCUSSION OF RESULTS

A stored-products insectary was established for the support of studies on potential insect induced toxicants in foods. Colonies of eleven of the most serious coleopterous pests were obtained from collaborators at the U.S. Department of Agriculture, Fresno, California, and the Department of Entomology, University of California, Riverside, California. Included in this group are five species of flour beetles (*Tenebrionidae*) which are known to secrete highly reactive *p*-benzoquinones. Selected military subsistence items are being used as the media for rearing. Physical conditions suitable for insect reproduction and development were optimized.

A gas-liquid chromatographic (GLC) method for the detection of three chemicals released by flour beetles was developed. The three chemicals are 2-methyl-1,4-benzoquinone (MBO), 2-ethyl-1,4-benzoquinone (EBQ), and 1-pentadecene (PD). The GLC method allows rapid and accurate analysis of levels of these chemicals in insects. The extraction of these chemicals from flour beetles is achieved with hexane/methanol (95:5). Investigations into methods for the extraction of these chemicals from flour have been unsuccessful to date.

Since benzoquinones have been reported as both acutely toxic and carcinogenic to laboratory animals, it was necessary to determine the amount of these compounds formed by flour beetles as a function of species, sex, and age. MBQ, EBQ, and PD concentrations were low (0.5-2.0 µg/insect) in newly eclosed *Tribolium confusum* and *T. castaneum*. The levels of these compounds increased with age until 20-30 days after eclosion, after which the concentrations maintained or declined slowly. Each 30-day female adult *T. confusum* contained mean concentrations of 20.1 µg MBO, 35.5 µg EBQ, and 24.2 µg PD. In



*T. castaneum*, each 30-day female adult contained mean concentrations of 21.3 µg MBQ, 21.3 µg EBQ, and 21.9 µg PD. For both species, adult females routinely contained higher levels of MBQ, EBQ, and PD than males of the same age.

Since current regulations allow *Tribolium* sp. infestations of three insects per 0.45 kg (1 lb) before condemnation of the subsistence, adult *T. confusum* or *T. castaneum* could contribute over 135 µg *p*-benzoquinone and 65 µg PD per kg of commodity. These amounts would be in addition to those secreted into the commodity by the beetles. During stress situations these compounds are rapidly released and replacement benzoquinones quickly synthesized. Investigations are continuing to obtain similar data for *T. madens* and *T. brevicornis*, which reportedly contain 3 to 5 times the concentration of *p*-benzoquinones found in *T. confusum* and *T. castaneum*.

#### CONCLUSIONS

An in-house resource of coleopterous stored-products pests of military subsistence was established for the first time at this Institute for the support of studies on potential insect induced toxicological problems in food. This capability has been expanded to include eleven species reared on selected military subsistence items. It was also possible to develop methods for detection of three chemical compounds released by flour beetles and to standardize and quantitate toxic materials released by some of these pests.

#### RECOMMENDATIONS

The investigation of *p*-benzoquinones and 1-pentadecene released by other species of *Tribolium* should be continued. Further work is also necessary on the release of these chemicals from flour beetles into infested commodities under different environmental and stress conditions. These data will provide information on the amount of *p*-benzoquinone present in infested flour which will be necessary for future toxicological analyses.

#### PUBLICATIONS

WIRTZ, R.A., S.L. TAYLOR, and H.C. SEMEY. *p*-Benzoquinone and 1-pentadecene concentrations in the flour beetles *Tribolium confusum* J. du Val and *Tribolium castaneum* (Herbst). Manuscript submitted for publication

EX-2      Benzoquinones from the secretory glands of *Tribolium confusum* and *Tribolium castaneum* - Assay, reactions, effects, and acute toxicity

### PROBLEM

The common flour beetles, *Tribolium confusum* and *Tribolium castaneum*, are major insect pests of stored-product subsistence. These flour beetles are known to produce two *p*-benzoquinones, 2-ethyl-1,4-benzoquinone and 2-methyl-1,4-benzoquinone, which have some reported toxicity. Therefore, stored-product subsistence could pose a military public health hazard. However, the reported toxicity of the *p*-benzoquinone is based on rather poorly designed experiments. While the *p*-benzoquinone may be toxic, the reaction of the *p*-benzoquinones with amino acids and proteins may modify that toxicity. The rate and extent of the reactions between *p*-benzoquinones and amino acids and proteins requires evaluation particularly in terms of stability. The reactions of benzoquinones with various food components and the resultant effects on food quality need to be assessed. The reactions of *p*-benzoquinones with important biomolecules may provide clues toward the biochemical basis of quinone toxicity. The acute toxicity of 2-ethyl-1,4-benzoquinones (EBQ) and flour to which EBQ has been added will be determined to allow comparisons of the toxicity of unreacted and reacted *p*-benzoquinones. Potential analytical techniques for the detection of these benzoquinones in food products will be evaluated.

### RESULTS AND DISCUSSION OF RESULTS

Preliminary investigations have shown that the product formed by reaction of proline with *p*-benzoquinone is not an unstable charge transfer complex as reported in the literature. The reactions between the *p*-benzoquinones and several amino acids were investigated, and spectral evidence would suggest the formation of several products including the 1,4-addition product. Research on the kinetics of these reactions is continuing.

### CONCLUSIONS, RECOMMENDATIONS, AND PUBLICATIONS

None; this study has not been completed.

STUDY NO. 21    Enterotoxigenic *Escherichia coli* from foods

EX-1            Assessment of methods for the identification of  
enterotoxin-producing *Escherichia coli* strains

### PROBLEM

The association of *Salmonella*, *Shigella* and *Staphylococcus aureus* with gastroenteritis is well-documented, and adequate methods for isolation and identification are available for these bacterial organisms. Recently, the potential of other bacterial species, such as *Escherichia coli*, to cause gastrointestinal disease has been recognized. Adequate methods for the separation and identification of

enterotoxin-producing *E. coli* (EEC) strains from strains not capable of enterotoxin production are not available. Although *E. coli* is routinely isolated from food products, the incidence of strains with enterotoxigenic properties is unknown. Since foods have been demonstrated as a vehicle of transmission for enterotoxigenic *E. coli*, the ability to identify such strains is important. Invasive and non-invasive EEC have been identified. Among the non-invasive strains which are frequently transmitted through foods and can produce diarrheal disease similar to salmonellosis, organisms may produce either an antigenic heat-labile enterotoxin (LT) or a non-antigenic heat-stable enterotoxin (ST), or both enterotoxin types. The ligated intestinal loop assay is the only method available for assay of ST, while several promising assays, such as the vascular permeability assay, activation of adenyl cyclase and its effect on tissue culture cells, and a serological assay have been developed for identification of LT-producing strains from clinical cases.

This experiment will evaluate the use of these methods for identification of EEC in foods as well as the feasibility for their routine use in food testing laboratories. An assessment of the incidence of EEC in foods will be made.

#### RESULTS AND DISCUSSION OF RESULTS, CONCLUSIONS, RECOMMENDATIONS

None; this study has not been completed.

#### PUBLICATIONS

None

EX-2      Methodology for isolation of EEC from foods

#### PROBLEM

Transmission of enterotoxin-producing *E. coli* (EEC) strains can occur through foods. An imported cheese was identified, in 1971, as the vehicle for the transmission of *E. coli* serogroup O124:B17 which caused an outbreak of gastroenteritis. At the time of that outbreak no precise methodology, other than serotyping, was available for the detection and identification of toxigenic *E. coli* biotypes. Following the imported cheese incident, a protocol, based on the use of elevated incubation temperatures, enrichment media, and serotyping of broths, was developed for recovery and identification of enteropathogenic *E. coli*.

Recent studies have revealed that no relationship exists between enterotoxigenicity and serotype. This correlates with the biological nature of plasmids and the demonstration that production of *E. coli* enterotoxin is plasmid-mediated.



*E. coli* is frequently isolated from foods. Examination of a beef soy product indicated a mean level of this organism in the product of 23/g. A random examination of enteric organisms in this product indicated six *E. coli* biotypes present. This is not an uncommon situation for a food product, however, a determination of enterotoxigenic capabilities of each biotype isolated would be time-consuming, expensive, and unfeasible for routine food testing laboratories.

A critical need exists for methodology for the selective isolation and identification of enterotoxigenic strains. This experiment will determine the conditions necessary for the isolation of enterotoxigenic *E. coli* strains from foods.

#### RESULTS AND DISCUSSION OF RESULTS, CONCLUSIONS, RECOMMENDATIONS

None; this study has not been completed.

#### PUBLICATIONS

None

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9. FRUIN, J.T., and W.S. CLARK, JR. Plate count accuracy: Analyst and automatic colony counter versus a true count. J Food Protect 40:552, 1977
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13. GUTHERTZ, L.S., J.T. FRUIN, R.L. OKOLUK, and J.L. FOWLER. Microbial quality of frozen comminuted turkey meat. J Food Sci 42:1344, 1977
14. FRUIN, J.T. (reviewer), Botulism, the Organism, Its Toxin, the Disease by L.D. Smith. Book review. JAVMA 171:288, 1977
15. GUTHERTZ, L.S., and R.L. OKOLUK. Comparison of miniaturized multitest systems with conventional methodology for identification of *Enterobacteriaceae* from foods. J Appl Environ Micro, In press
16. FOSTER, J.F., J.L. FOWLER, and W.C. LADIGES. A bacteriological survey of raw ground beef. J Food Protect, In press
17. FOWLER, J.L., W.S. CLARK, JR., J.F. FOSTER, and L.A. HOPKINS. Analyst variation in doing the standard plate count as described in *Standard Methods for the Examination of Dairy Products*. J Food Protect, In press

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6357	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB'N INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62172A	3M162172A811		00		005	
<del>XXXXXXXX</del>	62772A	3A762772A811		00		005	
<del>XXXXXXXX</del>	CARDS 114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Radioisotope Support for Military Medical Research							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008500 Isotopes; 013900 Radioactivity; 011000 Nuclear Instrumentation							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
64 05		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: Not Applicable				FISCAL YEAR		77	
c. TYPE:				CURRENT		1.2	
d. KIND OF AWARD:				78		31	
e. AMOUNT:				1.0			
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
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				Presidio of San Francisco, CA 94129			
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NAME: Canham, J.E., COL, MC				NAME: Morrissey, R.L., MAJ, VC			
TELEPHONE: 415-561-3600				TELEPHONE: 415-561-4770			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Military Research Projects; (U) Radioisotopes; (U) Instrumentation; (U) Data Acquisition							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objectives are to provide radioisotope support to all projects requiring the use of radioisotopes and improve procedures and counting techniques where needed, to conduct radioactive counting procedures for joint military medical research projects, to conduct research to improve technology, and to adapt existing technology to research areas of significance to the LAIR mission.</p> <p>24. (U) Methodology research is conducted as required to improve existing procedures. Ten automatic sample changing radiation detection instruments are maintained for detection of beta and gamma radiation. All aspects of the radiological protection program, as required by Nuclear Regulatory Commission licensure, are conducted.</p> <p>25. (U) 76 10 - 77 09 Research support has been provided to 34 investigators utilizing radioisotopes during this period. Support provided includes procurement and storage of radioisotopes, radiation safety monitoring, decontamination of glassware, radioactive waste disposal and maintenance of logs and records as required by Nuclear Regulatory Commission and Army regulations. Consultation concerning proper use and application of radioisotope technology has been provided as needed. Through the cooperative effort of the Health Physics Officer, LAMC, the Physicist, LAMC, and the Radiological Protection Officer, LAIR, a 32-hour training course entitled, "Safe Use and Handling of Radioisotopes", was presented in November 1976 and again in March 1977.</p>							



#### ABSTRACT

PROJECT NO. 3M16272811 Military Nutrition and Food Hygiene

WORK UNIT NO. 005 Radioisotope Support for Military  
Medical Research

Research investigators are currently being supported with radioisotope services, including procurement and storage of radioisotopes, radiation safety monitoring, decontamination of glassware, waste disposal, maintenance of appropriate logs and records, and maintenance of radiation detection instruments for investigator use. A 32-hour training course entitled, "The Safe Use and Handling of Radioisotopes," was presented on 2 occasions during the current fiscal year.

## BODY OF REPORT

WORK UNIT NO. 005

Radioisotope Support for Military  
Medical Research

### PROBLEM

The use of radioisotopes in biomedical research has proven useful. Radioactive substances may be used for tracer studies and therapy. Tracer studies may be utilized in either basic research or as diagnostic aids. Tracer procedures may be employed to follow the behavior of specific elements or compounds in the body, or to follow the path of any molecule to which a radioactive atom has been attached, when the latter takes no part in the metabolism. Thus, the formation of metabolic products can be traced, the utilization of metabolic products can be observed, and turnover studies can be readily performed. The Radioisotope Division is responsible for procurement and storage of radioisotopes, radiation safety monitoring, decontamination of glassware, radioactive waste disposal and maintenance of logs and regulations. Advice and counsel are given to investigators regarding the use of radioisotopes. Beta and Gamma counting instruments are maintained for use of investigators at LAIR in support of military medical research.

### RESULTS AND DISCUSSION OF RESULTS

Support functions delegated to the Radioisotope Division were maintained in the current fiscal year. Current instrumentation includes 4 gamma counting instruments and 7 liquid scintillation counters. When the Department of Information Sciences is able to provide the computer programming support, such as we previously had at the USAMRNL in Denver, we will be able to provide the services of disintegrations per minute determinations and additional mathematical calculations. Many hours of conference and planning time have been committed to this effort in this fiscal year. The system programming is estimated to be 75% completed.

Bulk procurement of radioisotope support supplies such as liquid scintillation counting solutions and vials results in price difference savings. Additional savings are accrued by processing a few large orders instead of numerous small orders from the 34 research investigators currently authorized to use radioisotopes. A charge back system has been established to distribute these costs to the appropriate research work unit.

Through the cooperative effort of the Health Physics Officer, LAMC, the Physicist, LAMC, and the Radiological Protection Officer, LAIR, a 32-hour training course entitled, "Safe Use and Handling of Radioisotopes," was presented in November 1976 and again in March 1977.

#### CONCLUSIONS AND RECOMMENDATIONS

The use of radioisotopes is essential to the mission of LAIR. It is recommended that the centralized support activity be maintained as the most economical and efficient means of making radioisotopes available to research investigators while maintaining adequate control of their use and thus protecting the health of laboratory personnel. Technological studies should continue to identify the most efficient, the most accurate, and the most economical laboratory procedures to be used in the routine use of radioisotopes.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
				DA OE 6080	77 10 01		
3. DATE PREV. SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DR&E INSTR <sup>a</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62172A	3M162172A811		00		010	
b. <del>CONTRACTOR</del>	62772A	3M762772A811		00		010	
c. <del>CONTRACTOR</del>	CARDS 114f						
11. TITLE (Precede with Security Classification Code) (U) The Metabolic Response of Hepatic and Extra-Hepatic Tissues to Dietary Substances, Drugs and Hormones in Health and Disease.							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 12		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE				PRECEDING			
b. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		4.0	
c. TYPE				CURRENT		166	
d. KIND OF AWARD				78		4.0	
e. CUM. AMT.						134	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME* Letterman Army Institute of Research				NAME* Letterman Army Institute of Research			
ADDRESS* Presidio of San Francisco, CA 94129				ADDRESS* Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J. E., COL, MC				NAME* Herman, R. H., COL, MC			
TELEPHONE: 415-561-3600				TELEPHONE: 415-561-4147			
				SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence not Applicable				NAME: Hagler, L., COL, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Hepatic Disease; (U) Hepatic Enzymes; (U) Enzyme Deficiency Disease, (U) Adaptive Responses of Hepatic Enzymes; (U) Laboratory Animals (U) Human Volunteers.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
<p>23. (U) Military personnel require highly responsive adaptive mechanisms for the regulation of blood glucose and other substances which are essential for normal mental and muscular activity. Previous studies have shown that certain hepatic enzymes adapt (i.e., increase or decrease in activity) to diet, folic acid, hormones, and medications. These adaptive responses maintain the blood glucose level by coordinating the processes of gluconeogenesis, glycolysis, glycogenesis, glycogenolysis, lipogenesis and lipolysis depending on the physiological state. Impairment of these complex adaptations may interfere with performance during training, combat operations, and recovery from injury.</p> <p>24. (U) The hepatic adaptive responses of selected patients with hypoglycemia and other disorders of blood glucose regulation and related problems will be studied. The effect of diet, folic acid, certain therapeutic agents, and exercise on blood glucose regulation will be studied. The acute effect of hormones on hepatic enzymes will be tested on animals.</p> <p>25. (U) 76 10 - 77 09 Hydroxocobalamin, a potent form of vitamin B<sub>12</sub> alleviated the subjective symptoms in 5 patients and improved the nerve conduction velocity measurements in 5 out of 7 patients with diabetic neuropathy. Glucagon increased and serotonin decreased blood glucose. Glucagon increased gluconeogenic enzyme activities and cyclic-AMP, whereas secretin decreased pyruvate kinase activity and cyclic-AMP. Cholecystokinin, histamine, serotonin, and gastrin increased pyruvate kinase activity. In one of three patients with hypoglycemia, hyperinsulinemia was found after glucose ingestion but not after intravenous glucose. Assays have been established to measure non-insulin sources of insulin-like activity which may account for glucose utilization in stress states.</p>							

# ABSTRACT

PROJECT NO. 3M762772A811 Military Nutrition and Food Hygiene  
WORK UNIT NO. 010 The Metabolic Response of Hepatic  
and Extra-Hepatic Tissues to Dietary  
Substances, Drugs and Hormones in  
Health and Disease

The following investigations have been conducted under this work unit:

STUDY NO. 7 The role of somatomedin and regulation of fuel for  
exercise

STUDY NO. 12 Studies of non-insulin dependent mechanisms of  
glucose utilization in exercise

STUDY NO. 7. The utilization and production of glucose during exercise is not completely understood. Glucose utilization increases while insulin secretion decreases and insulin is released from its receptor on exercising muscle. Insulin accounts for less than 10% of insulin-like activity (ILA) in serum as determined by bioassays. The remainder of the activity is due to substances collectively called non-suppressible insulin-like activity (NSILA). It is possible that one of these substances may be activated during exercise and may replace insulin as the mediator of glucose utilization. Somatomedin (Sm), a peptide produced in response to growth hormone (GH), exhibits ILA and can compete for the insulin receptor. Insulin, in theory, would have adverse effects on performance because it inhibits mobilization of free fatty acids from adipose tissue and inhibits hepatic gluconeogenesis. If Sm is the mediator of exercise glucose utilization, the effects on hepatic gluconeogenesis should be different than those of insulin. A radioreceptor assay has been developed in order to measure Sm levels in exercising humans (see Study No. 12, Work Unit 010) and to monitor its purification. Purified Sm will be used later to study the effects of Sm on hepatic gluconeogenetic enzymes and glucose production from <sup>14</sup>C-alanine in perfused livers.

STUDY NO. 12. Individual soldiers vary in their ability to exercise maximally or recover from injury. Both abilities are dependent partially upon the effective production and utilization of fuel substances like glucose. Such variations can possibly be explained by defects in the adaptive hormonal milieu that accompanies severe exercise or trauma. Somatomedin (Sm), which has insulin-like activity (see Study No. 7, Work Unit 010), may be involved in glucose utilization during stress states and probably plays a role in the reparative process. Measurement of Sm by a radioreceptor assay in 3 exercising subjects showed an increase in Sm activity and a fall to pre-exercise levels at the time of exhaustion in 2 experiments. Sm was slightly

elevated in the serum of a catabolic patient prior to starting parenteral hyperalimentation and rose even higher after beginning hyperalimentation. Plans are underway to study the acute and chronic changes in Sm that occur in normal soldiers undergoing a training program and in surgical or traumatized patients. Subsequent studies will focus on soldiers who perform poorly during basic training or maneuvers to compare the Sm adaptive response.



## BODY OF REPORT

WORK UNIT NO.	010	The Metabolic Response of Hepatic and Extra-Hepatic Tissues to Dietary Substances, Drugs and Hormones in Health and Disease
STUDY NO.	7	The role of somatomedin and regulation of fuel for exercise

### PROBLEM

The ability of soldiers to perform maximally, to endure physical and emotional stress and to recover from injury are, to a large extent, dependent upon the hormonal and metabolic adaptation that occur during such stress states. All of these functions are dependent upon fuel substances such as glucose, amino acids and free fatty acids which are derived from the diet or which are endogenously produced. One of the major fuel sources is glucose. The utilization and production of glucose during exercise and other stress states is not completely understood. Glucose utilization proceeds at an increased rate during exercise, while radioassayable insulin decreases and insulin is released from its receptor sites on exercising muscles. Diabetics can utilize glucose during exercise despite a total lack of insulin. These observations suggest that an alternative, non-insulin-dependent mechanism of glucose utilization, may be operating during such stress states. Failure to perform effectively during maximal exercise possibly could be due to defects in the non-insulin-dependent mechanisms of glucose utilization.

There are substances in the blood which clearly are not insulin but which have insulin-like activity. Insulin itself accounts for less than 10% of the total insulin-like activity of serum as determined by bioassays. One of the serum components which accounts for a significant portion of the total insulin-like activity is somatomedin (Sm). Sm is a growth factor, which is produced in response to growth hormone (which increases during stress states), is capable of exerting action on the insulin receptor, stimulates DNA synthesis, and stimulates other growth promoting enzyme reactions.

If Sm is responsible for non-insulin-dependent glucose utilization, one should be able to demonstrate an increase of Sm during exercise. Sm has been measured during exercise in one reported instance and despite the expected growth hormone increase, Sm did not increase. One possibility for the failure to see an increase in that experiment may have been the presence of a protease which rapidly degraded the Sm. This is a reasonable postulate since proteases are activated during exercise and teleologically such a substance should be subject of control or hypoglycemia might ensue after exercise. The action of insulin itself should be detrimental to exercise since it is a potent-inhibitor of lipolysis and gluconeogenesis. In theory, the substance which

replaces insulin during exercise should not inhibit hepatic gluconeogenesis which is a major source of glucose during exercise. One could indirectly test the assumption that Sm plays a role in exercise by examining the effect of Sm on hepatic gluconeogenic enzymes. Initial efforts at purifying sufficient Sm to perfuse isolated rat livers are underway in this laboratory. A system of perfusing isolated rat livers and the means to measure hepatic gluconeogenic enzymes are available.

#### RESULTS AND DISCUSSION OF RESULTS

A bioassay of Sm based upon serum stimulation of  $^{35}\text{SO}_4$  incorporation into cartilage fragments of hypophysectomized rats was set up in this laboratory. A previous report in the literature in which this assay was used showed no increase of Sm activity in the serum of an exercising subject. The effects of Trasylol<sup>(R)</sup> (a protease inhibitor) on the assay system were investigated. Trasylol<sup>(R)</sup> (2,500  $\mu\text{ml}$ ) caused significant depression of  $^{35}\text{SO}_4$  incorporation on base line values; i.e., cartilage incubated in buffer alone. Similar doses were without effect when added to incubation media containing cartilages and serum. In a preliminary study, Trasylol<sup>(R)</sup> was added to blood at the instant of collection from a healthy male subject before and after 30 minutes of exhaustive exercise. Serum Sm activity tended to be higher ( $p < 0.1$ ) at the end of exercise, but not significantly. The assay was variable and tedious to perform. Therefore, a radioreceptor assay of greater sensitivity and precision was developed with the assistance of Dr. K. Uthne, A. B. Kabi, Stockholm, Sweden. This assay is based upon the competition of Sm in an unknown sample with a  $^{125}\text{I}$ -radiolabelled trace (SmA, a neutral peptide) for receptor sites on crude human placental membranes. In trace binding to the membranes in 0.05 M Tris - 1% bovine albumin, the pH 7.4 varied between 8-24% depending upon the purity of the SmA used for labelling (lactoperoxidase method). Human serum incubated 18 to 20 hours at 4°C caused linear displacement at doses of 20, 40 and 80  $\mu\text{l}/300 \mu\text{l}$  reaction volume. Rat serum was approximately 7 to 10 times as potent in the assay as human serum with doses of 2.5, 5 and 10  $\mu\text{l}/300 \mu\text{l}$  incubation volume causing similar displacement. The displacement curves were parallel with the curves from a standard pooled serum from the same species which was arbitrarily assigned a value of 1.0 and from which the potency of unknown samples was calculated. This assay has been used to measure human Sm (see Study No. 12, Work Unit 010) and will be used to monitor Sm purification for perfusion of isolated rat livers.

#### CONCLUSIONS

The utilization of glucose during exercise and other stress states is likely due to the action of some hormonal substance other than insulin. An alternative mechanism involving Sm, which has insulin-like activity, has been postulated. The placental membrane radioreceptor assay is a valuable tool for determining small changes in Sm activity.

## RECOMMENDATIONS

Additional efforts at purification of sufficient quantities of Sm to perform liver perfusion studies should be completed. If Sm is found to augment gluconeogenesis in these animal studies, further studies to clarify the mechanism of Sm action during exercise and trauma should be undertaken with the possible goal that the effect of Sm could be potentiated pharmacologically to improve performance or improve the rate of wound healing in humans.

STUDY NO.	12	Studies of non-insulin dependent mechanisms of glucose utilization in exercise
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## PROBLEM

The utilization of glucose is generally considered to be dependent upon the presence of insulin. In some circumstances there appears to be significant non-insulin-dependent glucose utilization, e.g. during exercise, following some trauma, and during catabolic states. Since glucose is a major fuel for muscle work and central nervous system function it is conceivable that an abnormality in the non-insulin dependent mechanism of glucose utilization could lead to ineffective physical or mental performance during such stress states. Clearly, individuals vary in their ability to undergo severe physical activity or recover from trauma. It is probable that biochemical or hormonal factors may modulate the individual soldier's adaptation during the stress of exercise or trauma. Knowledge of the mechanisms by which substrates for energy are produced and utilized could provide a means for detecting individuals with limited adaptability during stress states.

We have developed a theory involving somatomedin (Sm) and possibly other factors to account for non-insulin-dependent glucose utilization during exercise (see Study No. 7, Work Unit 010). The same mechanism may be operative during other stress states, such as trauma, which are characterized by growth hormone secretion. The probable involvement of these substances during catabolic states is pointed out by studies of severely burned patients who have a significantly shorter period of negative nitrogen balance and an enhanced feeling of well being when treated with large doses of growth hormone. Growth hormone treatment also appears to prevent stress ulceration of the stomach, a common complication of severely injured patients. Since the anabolic response to growth hormone is mediated by substances like Sm it is probable that these substances are involved in the adaptation to stress states, which are characterized by an increase in hormone secretion. Treatment with growth hormone, however, is impractical because of limited amounts of the hormone. If the mechanism by which Sm and related substances are activated could be understood, it may be possible to provide a means of treating such patients more effectively.



## RESULTS AND DISCUSSION OF RESULTS

Using the placenta membrane radioreceptor assay for SmA which was more precise and less variable than bioassay systems, we have observed an increase in Sm activity during 60% maximal exercise in three subjects. In two of the three experiments in which exhaustion was achieved, a drop in Sm to the pre-exercise level was observed at the time of exhaustion. The significance of these findings is unknown. Work on the effect of protease inhibitors on the radioreceptor assay system has been started. It is possible that larger increases in serum Sm will be seen in exercising subjects if protease inhibitors are added immediately to collected blood. The fall in Sm activity at the time of exhaustion is interesting but more experiments must be done to determine if this is a reproducible finding. Using the same assay system, we measured Sm in one debilitated patient who was undergoing hyperalimentation. The Sm levels prior to hyperalimentation were slightly elevated, and increased further to clearly elevated levels after one week of hyperalimentation.

## CONCLUSIONS

The increased levels of Sm observed in exercising subjects and in a patient undergoing hyperalimentation suggest that Sm may be involved in the adaptation to stress states such as exercise and trauma.

## RECOMMENDATIONS

Additional studies are needed to clarify the mechanisms of non-insulin dependent glucose utilization during exercise and following trauma. Changes of Sm in subjects undergoing exercise and in patients in catabolic states should be more thoroughly investigated. The effects of protease and protease inhibitors upon the assay system are needed in order to elucidate the mechanism by which Sm and related substances are activated. Changes in Sm in a group of normal subjects during exercise and training can be applied to examine trainees and soldiers who fail to perform adequately during training or maneuvers. A medication should be sought which will activate the Sm system in normal subjects possibly to enhance exercise performance and in injured patients to enhance the recovery from injury.

## PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6081	77 10 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMRY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DDB'S INSTR <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM A. WORK UNIT
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62172A	3M162172A811		00		011	
<del>C. SECONDARY</del>	62772A	3M762772A811		00		011	
<del>D. TERTIARY</del>	CARDS 114f						
11. TITLE (Precede with Security Classification Code) (U) The Metabolic Responses of the Gastrointestinal Tract to Dietary Substances, Drugs and Hormones in Health and Disease.							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 12		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		77	
C. TYPE				CURRENT		78	
D. KIND OF AWARD				78		4.0	
E. AMOUNT				78		4.0	
F. CUM. AMT.				78		131	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J. E., COL, MC				NAME <sup>a</sup> Herman, R. H., COL, MC			
TELEPHONE: 415-561-3600				TELEPHONE: 415-561-4147			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Hagler, L., COL, MC			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Gastrointestinal Disease, Jejunal Enzymes, Adaptive Responses of Jejunal Enzymes, Diarrheal Syndromes; (U) Human Volunteers; (U) Laboratory Animals.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23.(U) Acute and chronic gastrointestinal (GI) diseases affect military personnel. These diseases cause long-term morbidity, loss of duty time; and high costs of continuing in- and out-patient care. In 1966 in Vietnam (RVN), diarrheal diseases accounted for 12% of all hospital admissions. In World War II functional dyspepsia and chronic idiopathic diarrhea accounted for 15-20% of admissions to war zone hospitals and 62% of these patients were returned to CONUS for treatment. Acute diarrhea is disabling and difficult to prevent since the etiology is often unknown (e.g. 87% of 1,954 patients in RVN, May 1965). Chloroquin, a potent anti-malarial agent, often causes acute diarrhea leading to non-compliance and hence ineffective malarial prophylaxis. GI injury from combat wounds precludes enteral feeding and results in atrophy of the small intestine, pancreas, and liver. Atrophy may be due to absence of gastrin which results from lack of enteral feeding or traumatic loss of gastrin secreting tissue. Gastrin replacement may reverse GI atrophy and enhance rehabilitation. 24.(U) We will study (1) the response of GI enzymes to diet, folic acid, and chloroquin in patients with chronic idiopathic diarrhea; (2) the effect of enterotoxins and chloroquin on animal jejunal mucosal enzymes in vitro; (3) the effect of gastrin administration on GI glycolytic enzymes in gastrin deficient patients; and (4) the ability of gastrin to enhance healing of GI injury in suitable patients. 25(U) 76 10 - 77 09 In an in vitro system, jejunal mucosa from the pig and guinea pig incorporated <sup>14</sup> C-leucine into protein, <sup>14</sup> C-thymidine into DNA, and <sup>14</sup> C-uridine into RNA. <sup>14</sup> C-leucine incorporation into protein was inhibited by cycloheximide. Preliminary in vitro studies indicate that pentagastrin infusion stimulated the incorporation of <sup>14</sup> C-leucine into human jejunal mucosa.							

# ABSTRACT

PROJECT NO. 3M762772A811 Military Nutrition and Food Hygiene  
WORK UNIT NO. 011 The Metabolic Response of the  
Gastrointestinal Tract to Dietary  
Substances, Drugs and Hormones in  
Health and Disease

The following investigations have been conducted under this work unit:

STUDY NO. 1 In vitro study of control of small intestinal  
mucosal enzymes

STUDY NO. 6 In vivo study of the control of small intestinal  
enzymes in man

STUDY NO. 1. It has been demonstrated that gastrin, an hormone elaborated by the antrum of the stomach and the first portion of the duodenum, has trophic action on the small intestine, liver, and pancreas of the rat. In order to study the effect of gastrin in vitro on human small intestinal tissue obtained by peroral biopsy, the in vitro techniques must be developed with the use of animal tissues. We have developed a technique for preparing suspension of rat small intestinal enterocytes which have at least 30 to 90% viability and less than 15% contamination with nonepithelial cells. The isolated enterocytes incorporate  $^{14}\text{C}$ -leucine into protein. Preliminary studies demonstrate that pentagastrin stimulates  $^{14}\text{C}$ -leucine incorporation into protein after 4 h of incubation.

STUDY NO. 6. Gastrin, a polypeptide hormone, synthesized in the gastric antrum, has recognized trophic effects in the small intestine in rats. Gastrin deficiency due to absence of enteral food or antral tissue leads to gastrointestinal atrophy. If the trophic effect of gastrin is required for normal intestinal protein synthesis, then gastrin deficiency (resulting from gastric or intestinal resection) will lead to abnormal protein and enzyme synthesis and subsequent abnormal gastrointestinal function. The acutely injured soldier with abdominal injuries requiring gastric or intestinal resection may be gastrin deficient and consequently postoperative gastrointestinal function may be favorably influenced by gastrin therapy. Nine patients were studied. We measured  $^{14}\text{C}$ -leucine incorporation into protein, and intestinal glycolytic enzymes pre- and post-pentagastrin infusion.



## BODY OF REPORT

WORK UNIT NO. 011

The Metabolic Response of the  
Gastrointestinal Tract to Dietary  
Substances, Drugs and Hormones in  
Health and Disease

STUDY NO. 1

In vitro study of control of small  
intestinal mucosal enzymes

### PROBLEM

Studies of the mechanism of action of gastrin, including its effect on  $^{14}\text{C}$ -leucine incorporation into protein, on the intestinal epithelial cell have been hampered by the fact that mucosal preparations contain a mixed population of cells including not only the intestinal absorptive cells but those of the lamina propria as well. In order to study the epithelial cells directly we have investigated the possibility of utilizing isolated enterocyte preparations. We have prepared viable rat intestinal epithelial cells, free of contamination by mesenchymal elements and gut flora.

### RESULTS AND DISCUSSION OF RESULTS

Our present method, which is a modification of many previous methods, has led to a procedure which is relatively simple, rapid, and results in an isolated villus cell population with 80 to 90% viability and less than 15% contamination with nonepithelial cells. Individual rat gut sacs filled with a 20 mg/dl hyaluronidase-sodium citrate-phosphate buffer were incubated at 37 C while being shaken at 100 cps in a Dubnoff shaker, then perfused with phosphate buffer. The perfusate was collected and centrifuged. The cellular pellet was collected, resuspended in phosphate buffer and centrifuged and resuspended in Trowell's basal salt medium with 10% calf serum added. To this cell suspension  $^{14}\text{C}$ -leucine was added and its incorporation into protein was determined.

Cellular viability was studied by staining with nigrosin, trypan blue, and 4-acetoamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), a fluorescent vital stain. Eighty to 90% viability was obtained after isolation with a diminution to approximately 70% after one hour.

Preliminary experiments in which isolated small bowel epithelial cells were used show  $^{14}\text{C}$ -leucine incorporation into protein. Early studies indicate that the addition of pentagastrin directly to the culture medium stimulates protein synthesis after four hours of incubation whereas, addition of chloroquin diphosphate appears to inhibit slightly, the incorporation of  $^{14}\text{C}$ -leucine into protein.

## CONCLUSIONS

It is possible to prepare relatively pure viable suspensions of enterocytes from the rat gastrointestinal tract. The enterocytes incorporate  $^{14}\text{C}$ -leucine into protein demonstrating that this is a suitable preparation for in vitro gastrointestinal studies. These results look promising.

## RECOMMENDATIONS

Further investigation should be done so that an in vitro enterocyte preparation can be utilized to study gastrointestinal hormone effects on protein and nucleic acid biosynthetic mechanisms particularly as affected by anti-malarial agents and enterotoxic agents produced by gastroenteritis-producing organisms.

## PUBLICATIONS

None

STUDY NO.	6	In vivo study of the control of small intestinal enzymes in man
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## PROBLEM

An important aspect of acute gastrointestinal disease involves combat-related abdominal injury and its sequelae. Abdominal injuries occur frequently in any military operation and develop serious complications. In World War II, in one field hospital, wounds of the stomach comprised 416 of 3,154 cases of abdominal injury. The fatality rate was 40%. Approximately 30% of the abdominal injuries consisted of wounds of the small intestine. Approximately 20% of the total number of injuries required partial resection of the gastrointestinal tract. Many patients with abdominal injuries will have altered gastrointestinal function secondary to resection of portions of the intestinal tract. With improved techniques of first aid, evacuation, blood replacement, surgery, and prophylaxis and treatment of infection, we can expect an increased number of combat-wounded soldiers to reach the postoperative period. At this point only general supportive measures are available and no specific therapy is known which can hasten healing and restore function of the gastrointestinal tract. Food intake, intestinal hormones and intestinal adaptation all make considerable contributions to the recovery process after intestinal resection. Several observations suggest that the antral hormone, gastrin, has trophic effects on the gastrointestinal tract. In rats, gastrin has increased  $^{14}\text{C}$ -leucine incorporation into protein,  $^{14}\text{C}$ -orotic acid incorporation into RNA, and  $^{14}\text{C}$ -thymidine incorporation into DNA. Gastrin trophic effects have been demonstrated in in vitro tissue cultures of rat gastric and duodenal mucosa. Pentagastrin stimulated epithelial cell growth, decreased cell doubling time, and decreased cell contact inhibition.

Two different laboratories have demonstrated the importance of food intake in regulating small intestinal enzymes. In rat, intravenous hyperalimentation decreased intestinal maltase and sucrase activities. Tissue gastrin fell concomitantly. The disaccharidases were restored to control levels by pentagastrin which suggests that gastrin may control intestinal disaccharidases returned to normal after oral feeding. Previous studies in this laboratory have demonstrated increased activity of jejunal glycolytic enzymes in response to carbohydrate meals. Specific sugars caused adaptive changes in the enzyme most concerned with the metabolism of the specific substrate and was in addition to a generalized increase in enzyme activity attributed to calories alone. Since food intake influences gastrin and intestinal enzymes, and since gastrin has documented trophic effects in the gut, it is conceivable that gastrin has a generalized effect on protein synthesis in the gut. If the trophic effect of gastrin is required for normal intestinal protein synthesis, then gastrin deficiency states, occurring as a consequence of gastric or intestinal resection, could result in abnormal protein synthesis and subsequent maladaptation of intestinal enzymes. There is ample in vivo and in vitro support for a gastrin trophic effect. There is also evidence to suggest that food intake is important in determining the level of intestinal enzymes and the amount of tissue gastrin. The acutely injured soldier who has lost variable amounts of stomach and small intestine has reduced intestinal function by virtue of the surgical resection. The enzyme activities in the remaining gut are responsive to food intake and gastrin, both of which have been reduced by the surgical procedure. It is reasonable to believe that replacement of gastrin will restore intestinal enzymes to normal and hasten restoration of gastrointestinal function. We will study the role of gastrin in the control of selected intestinal enzyme activities. Selected patients with surgically altered gastric physiology will be studied. We will determine if any alteration of the intestinal enzyme activity can be reversed by administration of pentagastrin. Related studies on the dog are being done (See Work Unit 043, Study No. 1).

#### RESULTS AND DISCUSSION OF RESULTS

To date 7 patients and 2 volunteers have been studied. There have been no technical problems related to pentagastrin infusion or obtaining jejunal tissue. The two volunteers did have considerable gastric secretion and delayed gastric emptying which caused nausea and vomiting. One volunteer was unable to tolerate the infusion for the full 16 hours and received only 64% of the calculated dose.

Measurement of jejunal tissue in vitro demonstrated that 5 out of 7 patients and both volunteers had increased incorporation of  $^{14}\text{C}$ -leucine into TCA precipitable protein. The data suggest that pentagastrin may increase  $^{14}\text{C}$ -leucine incorporation into protein. Measurement of glycolytic enzymes showed an increase in pyruvate kinase activities in the 3 patients who had Billroth-I surgery and one patient with idiopathic diarrhea. Increases were seen in fructose



diphosphate aldolase, fructose-1-phosphate aldolase, and fructose diphosphatase in the patients who had Billroth-I surgery. The glycolytic enzymes in the normal volunteers either did not change or decreased. No significant changes were seen in disaccharidase activities.

#### CONCLUSIONS

Our preliminary data suggest that pentagastrin increases the ability of jejunal epithelial cells of man to incorporate <sup>14</sup>C-leucine into protein in vitro and increases glycolytic enzymes in certain patients.

#### RECOMMENDATIONS

These results are encouraging and the studies should be pursued. More patients and volunteers should be studied. We have mastered many of the technical details of the study which should facilitate our future investigations; for example, we have shown that pentagastrin can be given intravenously with little or no untoward effects.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6116	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
77 07 19	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62172A		3M162172A812		00 001	
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11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Studies to Assure a Supply of Nonhuman Primates for Research							
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001700 Animal Husbandry; 012900 Physiology; 002600 Biology; 002300 Biochemistry							
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				NAME: Bucci, T.J., LTC, VC			
				NAME:			
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22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Primate; (U) Reproduction; (U) Animal Colony;							
(U) Physiology; (U) Behavior; (U) Husbandry							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) An assured supply of primates is essential for continued research to solve Army medical problems in infectious disease, laser standards, environmental quality standards, new drug development, and a variety of other areas. Curtailment of export by the Colombian source has eliminated the overseas supply of owl monkeys (Aotus) required for ongoing malaria research. Owl monkeys are nocturnal primates and there are few data on their requirements in captivity or on the behavior and needs of their offspring. India has reduced its supply of rhesus monkeys drastically. This species comprises 50% of all monkeys used in Army research. This work unit will develop information on reproductive biology, nutrition, disease control, and low-cost husbandry methods, including outdoor breeding colonies, and will establish optimal husbandry methods for domestic production of required nonhuman primates.							
24. (U) Owl monkey pairs will be matched by karyotype and housed in a variety of cage/group arrangements. Reproductive potential will be evaluated by observation and physiologic measurements. Rhesus monkeys will be housed outdoors in enclosures containing simulated troops of 50-100 monkeys, and low-cost husbandry methods will be compared.							
25. (U) 7610-7709 The owl monkey colony is composed of 80 animals. Successful husbandry methods for a breeding colony have been established and an economical homemade cage system has been adopted. Nineteen live young have been produced in 19 months by 14 pairs. Hematologic, physiologic, and behavioral studies are in progress. Sixty rhesus have been accumulated as a nidus for domestic breeding of this species; most are former research animals from other laboratories. They are being evaluated for breeding potential. Establishment by contract of an outdoor breeding colony of rhesus has been deferred through lack of funds.							

# ABSTRACT

PROJECT NO.	3M762772A812	Military Research Animal Resources
WORK UNIT NO.	001	Studies to Assure a Supply of Nonhuman Primates for Research

The following investigations have been conducted under this work unit:

STUDY NO. 1	Basic studies in reproduction of owl monkeys
STUDY NO. 2	Development of husbandry methods for an outdoor colony of macaque monkeys

Nonhuman primates are essential for a number of areas of military biomedical research. Most notably they are used in infectious disease to establish pathogenesis of infections and tests of chemotherapeutic agents and vaccines. They are also needed for research in trauma, resuscitation, performance, and toxicologic studies. All of these studies are designed to benefit military personnel who are exposed to disease or toxic substances, or who suffer injury in performance of duty.

Nonhuman primates from foreign sources are becoming decreasingly available; future supplies depend upon development of domestic breeding colonies. For macaque species, considerable data and some experience are available for management of breeding colonies of captive monkeys. Much needs to be learned so they can be produced in large numbers and as economically as possible.

An urgent need exists in malaria research for Aotus species (owl monkeys); they may soon be included on the list of endangered species and be virtually unobtainable from countries of origin. A limited amount of data are available about the husbandry of this nocturnal primate.

This Work Unit establishes protocols to gain information about efficient husbandry methods for macaque and owl monkeys.

STUDY NO. 1. At year's end there were 110 animals in the owl monkey colony, including 28 pairs matched by karyotype. Twenty offspring had been produced in 21.5 pair-years. Basic husbandry conditions and practices were established, and hematologic, biochemical, and behavioral data were accumulated and analyzed. Minimum conditions for operation of a laboratory breeding colony of Aotus monkeys have been defined.

STUDY NO. 2. To establish an economical outdoor breeding colony of macaque monkeys (rhesus and cynomolgus), a tract of land was reserved at Camp Parks, CA, but development was precluded by lack of funds. A Request for Proposal (RFP) was written to obtain extramural research in outdoor production of macaques, but the RFP has not been advertised



for the same reason. Ninety animals are on hand to be used as a nucleus for the program. They are being characterized for reproductive capability.

## BODY OF REPORT

WORK UNIT NO.	001	Studies to Assure a Supply of Nonhuman Primates in Research
STUDY NO.	1	Basic studies in reproduction of owl monkeys

### PROBLEM

There is a critical shortage of owl monkeys (*Aotus* sp.) for biomedical research because the countries of origin have severely limited exportation. In the near future owl monkeys will be placed on the list of endangered species. This will make importation of these animals impossible at any cost.

Owl monkeys are the only nonhuman primate in which falciparum malaria can be maintained. Consequently, the Army is dependent upon these animals for research in prevention and treatment of malaria.

The long term solution to provide a source of these animals is a domestic breeding program. Little is known about the reproductive biology or behavior of owl monkeys; until recently, consistent breeding and rearing of these animals in captivity had not been accomplished.

The objective of this study is to determine the environmental and husbandry conditions, physiological state, and behavior which contribute to successful laboratory breeding and rearing of owl monkeys. The specific investigations in progress are owl monkey husbandry, cytogenetic analysis, behavior patterns, hematology, blood chemistry, virology, pathology, growth, pregnancy diagnosis, nutrition, and urine analysis.

### RESULTS AND DISCUSSION OF RESULTS

The owl monkey colony now comprises 110 animals. In addition to newborns, a group of 30 wild-caught animals from Bolivia and another group of 30 from Panama were added to the colony in the last year.

A movable aluminum frame was designed and built to support the wire cages and nest boxes which we designed and tested last year. As the cages and nest boxes were constructed, paired animals were moved into them. All paired animals with their youngest offspring are currently housed in these cages. All juvenile animals are housed in larger cages of the same design. Single animals are housed in single stainless steel rabbit cages on cascade racks. This system seems to be working well. The labor required to clean the owl monkey area has been reduced by two-thirds even though there are twice as many animals.

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To approximate the animals' natural habitat, the animal rooms were maintained at 27 C and 50-60% relative humidity. There was a 12-hour light/dark cycle with a 30-minute transition period ("dawn" and "dusk"). Because Aotus sp. are nocturnal, normal husbandry procedures are carried out during the dark cycle (0900-2030 hrs) when illumination consists of dim red light (0.4 foot candles).

Nutrient requirements of this genus are not known, so we provided a variety of foodstuffs on a fixed schedule. The dietary constituents and the schedule are listed in Tables 1 and 2, respectively.

To reduce variables in the environment, access to the colony is restricted to the principal investigator who is a veterinary officer and 3 specific veterinary technicians.

Cytogenetic analysis of most of the owl monkeys (performed by Dr. T.C. Jones of Pathobiology, Inc., Marlboro, MA) has been completed. Results have not yet been received for some animals most recently imported from Panama and some LAIR-born juveniles. Two previously undescribed karyotypes were discovered in 4 animals purchased from Yale University. These are Type VIII ( $2n = 55$ ) and Type IX ( $2n = 56$ ). All monkeys imported from Bolivia are Type VI (male  $2n = 49$ , females  $2n = 50$ ). The 4 Panamanian animals typed are either Type II ( $2n = 54$ ) or Type III ( $2n = 53$ ). This may indicate that these animals are genetically similar to the Columbian owl monkeys.

There are now 28 pairs of monkeys matched by karyotype. To date 20 offspring have been produced by 12 pairs during approximately 21.5 pair-years. Eighteen pairs have either not produced offspring or have had a long interval between offspring. The following factors may contribute: (1) the animals do not have the optimum karyotype to produce viable gametes, (2) they are sexually immature, (3) they are physically disabled or impaired, (4) they have been paired too recently. Our success in raising healthy offspring demonstrates that wild-caught animals can be maintained and mated in the laboratory. However, establishment of a production colony requires that the offspring also mate successfully. We estimate that these animals must be 2-3 years old before they reach sexual maturity.

The acquisition of a low-light level television camera equipped with a remote-control zoom and pan/tilt mechanism, high resolution monitor, and recording equipment has permitted remote observation of owl monkey behavior during all phases of the light/dark cycle. The first phase of the study, to establish a behavioral taxonomy, has been completed. A behavioral taxonomy is a qualitative list of all behavior observed. The behavioral taxonomy was divided into interactive and noninteractive behavior. Categories included in noninteractive behavior were feeding, drinking, autogrooming, resting, elimination, locomotion, and other motor activity. Interactive behavior is divided into environmental (activities directed toward external stimuli but

excluding behavior directed toward other monkeys), social (activities directed toward other monkeys but excluding infant care), and infant care.

When the taxonomic list was completed, we began to assemble an activity profile, which lists the frequency of a specific behavior. There are 2 groups of animals in this phase of the study. One group consists of 12 pairs and the other is made of 15 juveniles in gang cages. Each animal is observed for a minimum of 8 ten-minute periods. Behavior is classified and recorded every 10 seconds during the 10-minute period. For both groups more than 20,000 observations will be recorded. A frequency matrix will be compiled by computer to reflect total occurrences of behavior by individuals.

Valuable management data have already been gained from these observations. For example infants have been observed to eat solid food at about 3 weeks of age; the male parent shares in the care of offspring; fighting is rare between members of a pair, but it occurs between pairs when their cages are too close together.

Data to determine normal blood values in owl monkeys have been collected. A specialized self-editing computer program has been designed to accept input by relatively untrained personnel via CRT terminal. A report form which permits easy visualization and verification of input data and manual data analysis has been designed. More complete analysis is performed by computer.

Since 1975 approximately 400 blood samples representing nearly all the animals have been collected. For initial analysis and to determine baseline hematologic values, blood was collected from 65 anesthetized, clinically normal animals. These animals comprised the following groups: 27 wild-caught adult females, 27 wild-caught adult males, 7 LAIR-born juvenile females, 4 LAIR-born juvenile males. Hematologic values from these 65 animals are contained in Table 3.

By comparing hemograms between clinically normal and abnormal animals we have gained useful knowledge of owl monkey hematology. For example, we have found erythrocyte sedimentation rate (ESR) to be a fairly accurate but nonspecific indication of disease in owl monkeys. The ESR and total leukocyte count together are particularly useful to distinguish sick animals. An idiosyncrasy of owl monkeys, compared to other species, is the marked increase in percent and absolute numbers of eosinophils present in both clinically normal and abnormal animals. The cause of this apparent eosinophilia is unknown.

To observe the effect of altered environment on the hemogram, we removed 2 groups of 3 animals each from the main colony area and placed them in another room. In this room the animals were subjected to an altered light-dark cycle, increased noise, and increased technician contact. All other environmental factors remained unchanged.

Within 3 days there was marked increase in the mean total leukocyte number and percent eosinophils and decrease in percent lymphocytes. In mean absolute count, lymphocytes decreased from about 4800/ $\mu$ l to 4100, while segmented granulocytes increased from 5100 to 6500, and eosinophils nearly tripled in number, 1200 to 3200/ $\mu$ l. All other hematologic values, both at the beginning of the experiment and after 3 days, were the same as mean values for the colony.

We believe this experiment demonstrates that mild stress can markedly alter the hemogram of clinically normal owl monkeys. This alteration may complicate interpretation of hemograms, particularly important in clinically abnormal or experimental animals.

Determinations of 15 serum chemical constituents have been made on a GEMSAC centrifugal fast analyzer. Serum was collected from clinically normal owl monkeys which had hematologic values within normal limits at the time of serum collection. The concentration of the serum constituents are listed in Table 4.

The following serum components are either within normal limits reported for another nonhuman primate (squirrel monkey) or are similar to values reported for one other group of owl monkeys: serum glutamic-oxaloacetic transaminase, serum protein, albumin, glucose, cholesterol, blood urea nitrogen, creatinine, sodium, potassium, and chloride. There was large variation in concentrations of alkaline phosphatase and lactic acid dehydrogenase between animals: hence, a value for "normal range" is of little use. Values for individual animals did not vary to the same degree in sequential samples, so it may be possible to establish individual normal values. Other causes for the "between-animal" variation could be differential effects of anesthesia, handling of the serum specimens, and variability in analyzer function. Uric acid values are slightly higher than those reported previously for owl monkeys. These differences could result from measurement techniques or differences in diet. Phosphorous values and calcium/phosphorous ratios vary between animals and between samples from the same animal taken at different times. Causes could include diet, food utilization, and physiologic status.

Swab samples from two sites, the oropharynx and rectum, were taken for virus isolation to identify the viruses commonly carried by this colony. The work was accomplished and reported under Project No. 3M762772A812, Work Unit No. 002, Study No. 3.

Growth and maturation of LAIR-born offspring are being documented with records of weight gain and dental development. This study was begun recently; definitive data have not been compiled. Results from this study will be helpful to estimate age of wild-caught monkeys and to evaluate development of future offspring.



An immunologic pregnancy-diagnosis technique has been tested and proven effective. The test is based on a qualitative assay for urinary chorionic gonadotropin. The nest boxes were easily adapted for urine collection; these help to minimize physical and psychological stress on a potentially pregnant female.

Since May 1975, 16 monkeys have died, most of them recently imported animals. All were necropsied, and tissues were evaluated by light microscopy. In some cases scanning and transmission electron microscopy were also used. Causes of death have been variable and unrelated. Notable, nonfatal lesions observed include aspermatogenesis in adult males, lymphocytic interstitial nephritis, and mesangio-proliferative glomerulitis. Clinically observed anemia resulting in death in several animals has been a frustrating problem.

#### CONCLUSIONS

1. Wild-caught owl monkeys can be maintained and mated feasibly in a controlled environment.
2. Owl monkeys are uniquely sensitive to alterations in their environment and respond in a variety of ways which interfere with good health and reproduction (and which would also introduce great variability in experimental data).
3. The husbandry conditions in our colony are appropriate and efficient.
4. Potential mates must be matched by karyotype for maximum productivity.
5. Hematologic and biochemical characteristics of these animals are poorly defined, and indices diagnostic of disease or other stress are not well established.
6. Nutritional requirements of this genus are not established.
7. Classification of behavior patterns is a valuable baseline of information for observing and communicating about the nonhuman primates and for colony management.

#### RECOMMENDATIONS

The provisions of the protocol should be completed, to include establishment of nutrient requirements, hematologic and biochemical reactions to specific stresses, and testing of pairs of offspring for fecundity.

#### PUBLICATIONS

1. KELLEY, S.T., R.S. MURRAY, and N.L. SAY. Establishment of an owl monkey breeding colony. (Abstract) In: Abstracts for the

27th Annual Session of the American Association for Laboratory  
Animal Science (Houston, TX, 7-12 Nov 1976)

2. ROSS, P.E., S. SILVERMAN, and T.J. BUCCI. Gastrointestinal transit of a barium meal in rhesus monkeys with or without history of gastric tympany: Effect of ketamine. (Abstract) In: Abstracts for the Inaugural Meeting of the American Society of Primatologists (Seattle, WA, 16-19 April 1977). p 52
3. KELLEY, S.T., and B.C. LEIBRECHT. Procedures for observing owl monkey behavior in a laboratory breeding colony. (Abstract) In: Abstracts for the Inaugural Meeting of the American Society of Primatologists (Seattle, WA, 16-19 April 1977). p 74
4. KELLEY, S.T., R.S. MURRAY, N.L. SAY, and G.S. WARD. Initial experience with a breeding colony of owl monkeys (Aotus sp.). Lab Anim Sci (submitted for publication)

TABLE 1  
Owl Monkey Diets

<u>Diet</u>	<u>Food Item</u>	<u>Wt. (g) per Portion</u>	<u>Comment</u>
A	Commercial monkey diet, dry	55	Soaked until soft Fed in stainless steel bowl.
	Instant breakfast drink	5	
	Water	45	
B	Frozen orange juice concentrate	25.3	Blended to a thick liquid consistency. Fed in stainless steel bowl.
	Sugar	24.1	
	Fresh bananas	19.5	
	Vitamin/mineral supplement	0.59	
	High calorie supplement	3.24	
	Baby cereal	13.35	
	Multiple vitamins	0.09	
	Eggs	5.4	
	Water	102.9	
C	Commercial monkey diet, canned	75	Provided in pieces. Fed in stainless steel bowl.
	Water	10	
D	Fresh banana	25	Cut into pieces Fed in stainless steel bowl.
	Fresh apple	50	
	Fresh orange	50	



TABLE 2  
Diet Schedule<sup>1</sup>

	<u>Sun</u>	<u>Mon</u>	<u>Tues</u>	<u>Wed</u>	<u>Thurs</u>	<u>Fri</u>	<u>Sat</u>
A.M. <sup>2</sup>	A	B	C	D	B	C	D
P.M. <sup>3</sup>	A	A	A	A	A	A	A

<sup>1</sup> Refer to Table 1 for composition of each diet

<sup>2</sup> A.M. feeding is about 0900 hours

<sup>3</sup> P.M. feeding is about 1530 hours

TABLE 3

## Hemogram of Clinically Normal Owl Monkeys

<u>n = 67</u>	<u>Value</u>	<u>+ 1 S.D.</u>
Erythrocytes ( $\times 10^6/\mu\text{l}$ )	5.82	0.29
Hemoglobin (g/dl)	14.27	1.21
Packed cell volume (%)	43.32	4.07
Leukocytes ( $\times 10^3/\mu\text{l}$ )	8.66	2.02
Band neutrophils (%)	0.28	0.79
Segmented neutrophils (%)	43.00	11.25
Lymphocytes (%)	48.36	11.84
Eosinophils (%)	6.19	8.13
Basophils (%)	0.10	6.39
Monocytes (%)	1.88	1.25
Erythrocyte sedimentation rate (mm/hr)	3.48	1.52
Plasma protein (g/dl)	7.03	0.16
Fibrinogen (mg/dl)	501.5	20.40

TABLE 4  
Hemogram of Stressed Owl Monkeys

<u>n = 6</u>	<u>Day 0</u>	<u>Day 3</u>
Erythrocytes (X 10 <sup>6</sup> /μl)	5.67	5.17
Hemoglobin (g/dl)	15.28	14.67
Packed cell volume (%)	45.58	43.85
Leukocytes (X 10 <sup>3</sup> /μl)	11.8	14.1
Band neutrophils (%)	0.33	0.33
Segmented Neutrophils (%)	43.17	45.83
Lymphocytes (%)	41.00	28.83
Eosinophils (%)	10.00	23.5
Basophils (%)	0	0.17
Monocytes (%)	1.70	1.33
Erythrocyte sedimentation rate (mm/hr)	3.83	3.33
Plasma protein (g/dl)	6.95	6.92
Fibrinogen (mg/dl)	499.16	491.67



## STUDY 2

### Development of husbandry methods for an outdoor colony of macaque monkeys

#### PROBLEM

There is a critical shortage of rhesus monkeys (Macaca mulatta) for research because the countries of origin have restricted exports. The rhesus monkey is used by the US Army for many areas of research. The cynomolgus monkey (Macaca fascicularis) is a possible replacement for the rhesus monkey, at least for some studies. However, the degree of interchangeability of the two species is not known. The cynomolgus monkey faces the same problems (i.e., about restrictions on exports) that have resulted in scarcity of rhesus monkeys. The Army must provide for its own future needs for these species and must do so in the most expeditious and inexpensive fashion possible. There is also the real danger that no breeding stock will be available after the next 2 to 3 years.

The best solution is to establish domestic breeding colonies of these species. In order to do so, inexpensive, efficient, and scientifically sound methods of husbandry, medical management, and facility design must be developed. In the case of the rhesus monkey, considerable information is available, but little is known about the specifics of outdoor breeding colony operations. The cynomolgus monkey has seen only limited use in this country as an experimental animal. Little is known of its reproductive biology or climatic adaptability.

The object of this study is to develop inexpensive techniques of outdoor colony management, husbandry methods, disease control and eradication, breeder selection, and rearing of offspring. For the cynomolgus monkey, ovulatory cycles, hormonal cycles, and social factors beneficial to reproduction must be discovered.

#### RESULTS AND DISCUSSION OF RESULTS

Most of the 71 rhesus and 19 cynomolgus monkeys on hand are juveniles. They have been observed for onset of sexual maturity and for distinctive behavioral patterns. To conserve resources, clinical laboratory examinations have been restricted to the minimum necessary to protect the health of the colony. Less than half the rhesus females have regular menstrual cycles, but there has been a marked improvement in regularity over the past nine months. As a check for breeding maturity and the ability of males to copulate, some in-cage breeding has been started. This has resulted in one birth, three abortions, and two current pregnancies. Three females and one male, all about 12 years old, were born in the United States and cage-raised. They have been studied in particular to determine how their history may affect breeding suitability. Only one of the females has regular menstrual cycles. All 3 have been mated at least once, with no resultant pregnancies. The 3 females appear to

copulate normally, but the cage-raised male has been removed from the breeding program after repeated refusal to copulate. This animal exhibits many stereotyped behavior patterns, such as pacing, which are not interrupted by the presence of a receptive female.

We were directed by HQ, USAMRDC, to prepare a Request For Proposal (RFP) to solicit bids for an extramural contract program to breed macaque monkeys outdoors. The RFP was submitted in July 1976, but it has not been advertised because of fund shortage.

#### CONCLUSIONS

As the community of investigators in the US who use nonhuman primates responds to the importation limitations and the cost of laboratory-raised and even laboratory-conditioned animals increases, use of nonhuman primates will be reduced to the minimum requirement. The Army requirement for macaque monkeys has not decreased. Nonhuman primates are still indispensable for on-going research in malaria and other parasitic and viral diseases.

Many questions remain unanswered about the suitability and selection criteria for relegating to breeding colonies older animals which have spent years in captivity. Yet attempts must continue to be made to salvage as many potential breeders as possible. Because macaques must be 3-5 years old to attain breeding proficiency and their offspring should be 3-5 years old to be placed on experiments (to preclude use of a juvenile population), a breeding program to produce animals for research requires a 5-7 year lead time. Each year lost postpones the day when MRDC might be self-sufficient in supply of nonhuman primates.

#### RECOMMENDATIONS

A continued supply of nonhuman primates for required research must be assured. This can be achieved by (1) appropriate agreement in conjunction with other national agencies via the Primate Steering Committee (NIH), (2) by in-house development of facilities and staff, (3) by extramural contract. The current program at LAIR to identify and retain breeders for future use should be continued.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6117	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA CONTRACTOR ACCESS <sup>a</sup>	8. LEVEL OF SUM <sup>a</sup>
77 07 19	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62172A	3M16217A812		00		002	
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				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Animal Model; (U) Physiology; (U) Animal Diseases; (U) Laboratory Animal							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH <sup>a</sup> 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) An estimated 75% of USAMRDC's budget is for studies in which laboratory animals are used. Animals have threefold importance as resources in medical research. (1) New knowledge of physiologic processes in soldiers can be derived through study of similar processes in the animal by using methods not feasible in man. It is imperative that the animals be in normal health and also that the correct animal species be used because interspecies variations occur. (2) Many diseases of animals may be studied as models to enhance prevention, detection, and treatment of counterpart diseases acquired by soldiers. (3) The animals are a major economic resource so there is constant need for improved husbandry methods. These studies will contribute to improved animal care and to identification, development, and use of animal models to investigate normal and abnormal health processes in soldiers.</p> <p>24. (U) Animal diseases which also occur in soldiers, such as dermatitis, will be characterized by modern laboratory methods to determine their appropriateness as model systems for the human condition. Costly laboratory animal diseases, e.g., respiratory disease in cats, will be studied for eventual eradication. Normal processes, e.g., utilization of vitamin C, will be studied so as to improve animal care as well as for possible extrapolation to the soldier.</p> <p>25. (U) 7610-7709 To characterize the prevalence of viruses in the LAIR animal colony, 240 viral isolates were obtained from the first 800 oral and rectal swabs taken from monkeys and cats. Known presence of specific viruses permits measures to be taken to minimize loss from disease and from ruined studies. Guinea pig losses from Bordetella pneumonia were shown to be unrelated to ascorbic acid nutrition. Blood gas characteristics of normal dogs were established under a variety of clinical conditions. Studies are ongoing to establish physiologic characteristics of swine as subjects to replace dogs and nonhuman primates for many applications.</p>							

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# ABSTRACT

PROJECT NO.	3M762772A812	Military Research Animal Resources
WORK UNIT NO.	002	Development of Military Research Animal Resources

The following investigations have been conducted under this work unit:

- |             |   |
|-------------|---|
| STUDY NO. 1 | Ascorbic acid and its effects on <u>Bordetella bronchiseptica</u>                         |
| STUDY NO. 3 | Infectious disease surveillance of laboratory animals                                     |
| STUDY NO. 4 | Skin graft rejection by Mexican hairless dogs   |
| STUDY NO. 5 | Physiological and biochemical characteristics of domestic swine used in military research |

More than 70% of HQUSAMRDC's budget is spent on studies which in some phase depend on use of animals. This work unit is directed towards conservation of the funds used in those studies by addressing improved animal health and husbandry and choice of appropriate model species.

STUDY NO. 1. In this study the possible relationship in guinea pigs between dietary ascorbic acid and susceptibility to pneumonia from Bordetella bronchiseptica was sought. Although the study design was appropriate, shortcomings in execution of the experiment resulted in data which were inconclusive.

STUDY NO. 3. This study revealed the kind and extent of viral infection(s) present in LAIR's feline, owl monkey, and rhesus monkey colonies. The study provides specific data on identity, prevalence, and incidence of certain viral agents. It provides important information to investigators regarding choice of animals for specific studies, possible explanations of morbidity and mortality patterns, and possible avenues for control/eradication of the infections. It also provides data necessary to make decisions about occupational safety of laboratory workers. The study showed feline herpesvirus, feline calicivirus, and feline syncytia-forming virus (FeSFV) to be widespread in cats from licensed dealers. These agents were shed for months, so that seemingly healthy cats released from laboratory quarantine can jeopardize uninfected cats elsewhere in the laboratory. The FeSFV is suggested to be associated with urinary and systemic disease. Special procedures were developed to culture FeSFV. Owl monkeys are common hosts for adenoviruses and infected animals shed Types II and III for many months. Offspring appear to become infected with Type III at an early age. The persistent infection produced by this agent may have a role in renal disease.

STUDY NO. 4. This study was a pilot study to demonstrate immune deficiency in a colony of hairless dogs; technical failures precluded definitive conclusions.

STUDY NO. 5. Swine have theoretical advantages over dogs and monkeys for certain biochemical and physiological studies because they are abundantly available in disease-free and genetically defined stocks, they are relatively inexpensive to maintain, and they do not have disproportionate ecologic or emotional factors associated with their use. This study is the beginning of a series to characterize swine for biomedical studies referable to the soldier. Blood gas and blood acid-base characteristics of swine are more similar to those in humans than those characteristics in dogs.

## BODY OF REPORT

WORK UNIT NO.	002	Development of Military Research Animal Resources
STUDY NO.	1	Ascorbic acid and its effects on <u>Bordetella bronchiseptica</u> in guinea pigs

### PROBLEM

Bordetella bronchiseptica can be isolated from the respiratory tract of apparently healthy guinea pigs in conventional colonies. Although frequently associated with pneumonia and other respiratory tract infections, it is usually considered to be an opportunistic organism. Partially depending upon such stress factors as malnutrition, crowding, prolonged temperature extremes, pregnancy, and experimental manipulations, the animals may either develop a carrier state or overt signs of illness. A lowered resistance of the animal may upset the balance of the carrier state.

Evidence of a correlation between ascorbic acid deficiency and lowered resistance to bacterial infection has been reported. It is possible that suboptimal dietary intake of ascorbic acid by guinea pigs is one factor which contributes to the puzzling sporadic outbreaks of bacterial disease that occur in apparently stable guinea pig colonies.

Guinea pigs are among the most important laboratory animals. In 1974, more than 600,000 were used for research in the United States; approximately 3,700 were used at LAIR in FY 75, when this study was planned.

Sporadic disease in guinea pigs is a major cause of animal deaths and of wasted research resources. At LAIR, 15% of 108 spontaneous deaths during CY 75 were caused by bacterial pneumonia. To begin to define this waste of resources, this study was undertaken to provide objective data about the specific interaction between dietary ascorbic acid and infection by Bordetella bronchiseptica in guinea pigs. While a correlation between ascorbic acid deficiency and lowered resistance to bacterial infection has been reported, conflicting data are also available. This controversy may be due to variables that were not controlled properly. This investigation was based upon quantifiable considerations of bacterial exposure and dietary ascorbic acid intake by guinea pigs housed in a defined environment.

### RESULTS AND DISCUSSION OF RESULTS

The study design called for Bordetella-free guinea pigs to be stabilized at three levels of dietary ascorbic acid. This was achieved by feeding an ascorbic acid-free synthetic diet for 21 days, then supplementing the



individual animals via oral pipette with ascorbic acid supplied daily in 20% sucrose solution.

The animals were maintained at constant body weight for 10 additional days by supplying them minimal ascorbic acid (approximately 0.25 mg/100 g of body weight per day) titrated against weight gain/loss. They were weighed and supplemented daily. Chronic scurvy thus was established; three groups of 18 animals each were established and fed 0.25, 2.5, and 18 mg ascorbic acid/100 g of body weight per day respectively via the pipette. A fourth group of 18 was being maintained on commercial guinea pig ration which supplied 1 mg ascorbic acid/100 g of body weight per day (diets and serum were analyzed periodically for ascorbic acid content). After 22 days at the prescribed intakes for ascorbic acid the animals were exposed to calibrated aerosols of viable pathogenic Bordetella bronchiseptica organisms, courtesy of the US Navy Bioscience Laboratory, Oakland, CA. Within each of the four animal groups, subgroups were exposed to the following numbers of organisms: none, 95-120, and 210. (A pilot study had revealed that 100 organisms were sufficient to establish infection in normally-fed guinea pigs.)

After exposure to the organisms the animals were maintained on their respective levels of ascorbic acid intake for 14 days. All survivors were killed and the number of B. bronchiseptica organisms recoverable from their lungs was quantified. The number of organisms present was to be the measure of their resistance to infection, and correlation was sought between the exposure dose, the ascorbic acid intake, and the number of organisms present 14 days after exposure.

There was no correlation between number of B. bronchiseptica recovered and either the exposure dose or the ascorbic acid intake of the animals. The organism was recovered from 43 of 48 survivors of the exposure, but from none of the 10 animals which were sham-exposed.

Superficially the data appear to provide reason to reject the hypothesis that a correlation exists between ascorbic acid and resistance to B. bronchiseptica. Critical audit of the raw data suggests instead that the experimental procedures as performed left much to be desired, and the data are thus indeterminable.

#### CONCLUSIONS

The protocol specified for this study could provide quantifiable data about the relation between ascorbic acid nutrition and resistance to bacterial infection. As performed, this study neither confirmed nor rejected the relationship, and the question is not resolved, although it remains an important one.

#### RECOMMENDATIONS

This study should be repeated with close adherence to protocol.

## PUBLICATIONS

None

STUDY NO. 3

Infectious disease surveillance  
of laboratory animals

## PROBLEM

Laboratory animals represent a major economic resource in the US Army medical research effort. Outbreaks of infectious disease in an animal colony are economically wasteful and can be devastating to on-going research. Monitoring for the presence of infectious agents through clinical histories, laboratory tests, immunologic surveys, studies of gross and histopathology, and microbiological studies are effective methods for the early recognition, diagnosis, and control of infectious diseases.

The objectives of this study are (1) to investigate the prevalence, cause, control, and elimination of infectious diseases among laboratory animals at LAIR, (2) to identify and characterize animal models of human disease from instances of spontaneous disease occurrence in the colony, (3) to identify and eliminate animals carrying dangerous zoonotic diseases.

Efforts were concentrated on prevalence of viruses in the cat colony and the owl and rhesus monkey colonies. General experience in many laboratories is that nearly 1/3 of dealer-supplied cats die of infectious diseases before being issued to investigators. We also began to characterize the viruses prevalent in our owl monkey colony.

## RESULTS AND DISCUSSION OF RESULTS

Feline viruses. Forty female cats, that were obtained from a commercial supplier in lots of 10, 4, 16, and 10, were monitored for respiratory viruses. Feline herpesvirus (FHV) was isolated from 30 of 40 cats (50 of 284 oropharyngeal swabs). Feline calicivirus (FCV) was isolated from 11 of 40 cats (28 of 284 swabs). Feline syncytia-forming virus (FeSFV) was isolated from 13 of 40 cats (82 of 284 swabs). Signs of severe respiratory disease usually occurred within the first 2 weeks of arrival and were associated with FHV infections. In the group of 16 cats severe respiratory disease was not observed until several months after arrival and subsequent to the introduction of a cat from another group. This cat was a chronic shedder of FHV. Four cats were chronic shedders of FHV and 6 cats were chronic shedders of FCV. All 13 cats that carried FeSFV were chronically infected. In 12 of 100 oropharyngeal swabs from FeSFV-infected cats, the presence of FHV or FCV destroyed the cell cultures and precluded detection of FeSFV. Disregarding these 12 swabs, FeSFV was recovered from 93% (82 of 88) of the remaining ones from these 13 cats. The isolation of FeSFV requires actively dividing cells for its expression. Inoculated cultures

of feline kidney cells were trypsinized 3 days after inoculation, and slide cultures were prepared. These slide cultures were stained after 2 to 3 days to demonstrate the multinucleated cells. The FeSFV was also isolated from buffy coat cells by cocultivation. The virus was isolated from the 13 infected cats during the first few days after arrival and was still recoverable in 5 of them 8 months later, in oropharyngeal swabs or buffy coat cells. Organ cultures of nasal and tracheal epithelium were prepared from one FeSFV-infected and one non-infected cat. The organ cultures of tracheal epithelium from both cats remained viable (motile cilia) for 6 to 8 months. The FeSFV was recovered from the tracheal cultures of the infected cat after 8 months in vitro.

In a test of vaccination efficiency, oropharyngeal swabs and blood were collected from 10 cats on the day they arrived, and 5 cats (No. 66, 68, 70, 72, 74) were vaccinated with Norden's "On the Nose" modified live vaccine that contains both FHV and FCV. The results of virus isolation attempts from oropharyngeal swabs are presented in Table 1.

Each of the 3 feline viruses (FHV, FCV, and FeSFV) were isolated from at least one cat prior to vaccination with FHV and FCV (Table 1). Following vaccination FHV was isolated 6 times: twice from one vaccinated cat, once each from 3 other vaccinated cats, and once from a non-vaccinated cat. FCV was isolated 13 times from 4 vaccinated and 2 non-vaccinated cats. The persistence of FCV was not prevented by vaccination in cat number 72. Six of the 10 cats were chronically infected with FeSFV. Only one cat had a significant neutralization antibody titer to FHV (Table 2). Eight of 10 cats had high levels of neutralizing antibody titers to FCV prior to vaccination (Table 3). However, high levels of antibody did not preclude persistent shedding of FCV in 3 cats.

Beneficial effects from vaccination were difficult to substantiate. Eight cats exhibited mild to marked signs of upper respiratory disease during the first 2 weeks of quarantine. One vaccinated (No. 74) and one non-vaccinated (No. 69) cat exhibited marked depression and anorexia which required forced feeding via stomach tube. Both cats were infected with FeSFV. Vaccination did not preclude intermittent shedding of FHV in cat No. 66 or persistent shedding of FCV in cat No. 72.

Of all 40 cats, one was killed because of severe respiratory disease (FHV) within 7 days after arrival. Three cats died acutely 10 to 12 days after arrival and had histopathologic lesions of feline panleukopenia. Two cats (Nos. 41 and 59) died at 59 and 80 days after a prolonged illness with depression, anorexia, and marked weight loss. Gross and histopathologic lesions indicated bacterial septicemia as the cause of death. The clinical signs were similar to those of cats Nos. 69 and 74 described above. These 4 cats were chronically infected with FeSFV. Although FeSFV infection has not been associated with one specific disease, it has been associated with chronic debilitating diseases: feline lymphosarcoma, feline infectious peritonitis, and urolithiasis. In 1974-75, 15 of 28 male cats being used for research at LAIR had



urolithiasis. Serum samples from 20 of 21 of these male cats contained FeSFV antibody.

By agar gel immunodiffusion 42 of 43 serum samples from the 13 FeSFV-infected cats were positive for FeSFV antibody. The 27 FeSFV-free cats were negative. The effect of FeSFV on the immune system of the cat has not been defined.

Owl monkey viruses. From a colony of 110 owl monkeys (*Aotus sp.*), 110 virus isolations have been made from oropharyngeal and rectal swabs. All isolates have not been characterized, but most of the isolates appear to be one of 3 types of adenoviruses. One isolate is a reovirus and 2 other isolates have not been characterized.

From a group of 30 owl monkeys newly imported from Bolivia there were 8 isolates of an adenovirus from oropharyngeal or fecal swabs of 7 animals. All isolations were from swabs collected during the first 2 weeks after their arrival at LAIR. Three animals died during this time. The virus was recovered from 2 of the 3 via swabs collected after deaths. Swabs from the third were contaminated with a yeast which precluded virus isolation. This agent was not recovered after the initial 2-week period despite repeated sampling thereafter. These 8 isolates agglutinate rhesus red blood cells (RBCs) but not human or rat RBCs. They replicate in owl monkey kidney (OMK) cells but not in primary rhesus monkey kidney, Vero cells, or feline kidney cells. In OMK cells, intranuclear inclusions are produced. The virus will pass a 0.1  $\mu$  filter but not a 0.05  $\mu$  filter. It is chloroform resistant and stable at 50 C for 30 min. Two of these isolates were sent to the Southwest Foundation for Research and Education (SWFRE), WHO Reference Center for Simian Viruses, San Antonio, Texas. These isolates, provisionally designated Owl Monkey Adenovirus Type I, were neutralized by antiserum to Squirrel Monkey Adenovirus Type I.

A second owl monkey adenovirus was isolated 38 times from 21 animals. Four of these isolates were from oropharyngeal swabs and 34 from fecal swabs. All except 4 of these isolates were from the 30 Bolivian monkeys. Two isolates were from recently acquired owl monkeys from Panama, and one each was isolated from a monkey acquired earlier from another laboratory and from a young animal that was born at LAIR. In OMK cells, all of these isolates cause similar cytopathic effects (CPE) with intranuclear inclusions. Selected isolates did not produce CPE in primary rhesus monkey kidney, Vero or feline kidney cells. This virus does not agglutinate rhesus RBCs. It passes a .1  $\mu$  filter but not a .05  $\mu$  filter, is chloroform resistant, and is stable at 50 C for 30 min. This agent, provisionally designated Owl Monkey Adenovirus Type II, was sent to SWFRE and was neutralized by antiserum to SV-11 virus, which is an adenovirus of rhesus monkeys. However, this isolate did not grow in LLC-MK<sub>2</sub> cells which are reported to be the cell of choice for SV-11 virus.

A third Owl Monkey Adenovirus, provisionally designated Type III, was isolated 67 times from 49 animals. All except 2 of these isolates were from oropharyngeal swabs. These isolates were from animals of all sources including 6 born at LAIR. This virus has not been well characterized because it requires 10 to 30 days to produce CPE and replicates only to low titers ( $1 \times 10^2$  to  $1 \times 10^{3.5}$ ). It produces CPE only in OMK cells. It replicates better in primary or low passage OMK cells than in established OMK cell lines.

Multiple isolations of Owl Monkey Adenovirus Types II and III were frequently from the same animal, separated by 3 to 6 months. On several occasions these 2 viruses were isolated at the same time from the same animals. All 3 adenoviruses were isolated from one animal within a 3-month period.

Production of immune rabbit sera to selected isolates of each type is in progress to confirm the identity of the isolates in each group. Virus neutralization tests will be performed on owl monkey sera to determine the immune status and the extent of the spread of these adenoviruses within the colony.

Rhesus monkey viruses. Virus isolation was attempted with oropharyngeal and fecal swabs from 126 animals. The following viruses were found: adenovirus - 10 (fecal); enterovirus - 3 (fecal); syncytia-forming virus - 1 (oropharyngeal). The serotypes of these isolates have not been identified. Selected tissue and body fluids collected at necropsy were tested, for virus with negative results.

## CONCLUSIONS

### Feline

1. FHV, FCV, and FeSFV are widespread in cats from licensed dealers.
2. Special tissue culture procedures are required to recover FeSFV from feline specimens.
3. One commercial vaccine was not effective in eliminating FCV and FHV from a small number of cats studied; some cats shed virus persistently despite high titers of antibody.
4. There is continued circumstantial evidence that FeSFV infection plays an important role in morbidity and mortality of laboratory cats. It appears to be associated with both urinary tract and systemic diseases.
5. Persistent shedding of these agents represents a hazard to established groups of cats when new seemingly healthy animals are introduced.

#### Owl Monkey

Owl monkeys are commonly hosts for adenoviruses and this group of viruses is the predominant one recovered from them. Owl Monkey Adenovirus Type III is widespread throughout the colony, and offspring appear to be infected early in life. Infected animals shed Adenovirus Types II and III for considerable periods.

#### Rhesus Monkey

None

#### RECOMMENDATIONS

##### Feline

1. Systematic study of the virulence and pathogenesis of FeSFV should be undertaken to determine its precise role in disease and loss of laboratory cats.
2. Continued surveillance is indicated to inform investigators of the status of their cats with respect to these agents.

##### Owl Monkey

Surveillance should continue to document the spread and prevalence of the adenovirus; development of immunity and possible resistance should be studied. The role of these agents in disease should be explored.

##### Rhesus Monkey

1. Viral surveillance of the rhesus colony should be continued.
2. Serotypes of viruses isolated should be identified.

#### PUBLICATIONS

1. SHROYER, E.L., and M.R. SHALABY. The isolation of feline syncytia-forming virus from oropharyngeal swabs and buffy coat cells. Am J Vet Res (in press)



TABLE 1

Virus Isolations From Oropharyngeal Swabs

Cat No.	Days After Arrival at the Laboratory						
	0	5	12	26	47	68	105
66	-	A	-	-	-	NT	A
67	B	B	B	B	B	B	NT
68	A	C,B	B	B	B	B	NT
69	B	B	B	B	B	B	NT
70	-	A	B	B,C	B	B	NT
71	B	B	B	A	B	B	NT
72	-	C	A	C	C	-	NT
73	C	C	C	C	C	C	NT
74	C	A,B	B	B	B	B	NT
75	-	C	C	C*	NT	NT	-

A = Feline herpesvirus (FHV)

B = Feline syncytia-forming virus (FeSFV)

C = Feline calicivirus (FCV)

NT = Not Tested

- = No Virus Isolated

\*Day 36

TABLE 2  
Virus Neutralization Titers to FHV

<u>Cat No.</u>	<u>Days After Arrival at the Laboratory</u>		
	<u>0</u>	<u>26</u>	<u>68</u>
66	<2	<2	NT
67	<2	<2	<2
68	16	64	16
69	<2	<2	<2
70	<2	4	2
71	<2	2	2
72	<2	<2	2
73	<2	<2	<2
74	<2	2	2
75	<2	<2	NT

TABLE 3  
Virus Neutralization Titers to FCV

<u>Cat No.</u>	<u>Days After Arrival at the Laboratory</u>		
	<u>0</u>	<u>26</u>	<u>68</u>
66	160	2560	NT
67	2560	>5120	>5120
68	2560	>5120	>5120
69	<5	10	5
70	>5120	>5120	>5120
71	>5120	>5120	2560
72	<5	1280	1280
73	>5120	2560	1280
74	>5120	>5120	>5120
75	>5120	>5120	NT



STUDY NO. 4

Skin graft rejection by Mexican  
hairless dogs

#### PROBLEM

This was a pilot study to determine whether the so-called "Mexican hairless" dogs maintained by the Department of Dermatology Research have an immune deficiency. They were to be tested by measuring their rate of rejection of homografts. Documentation of immune deficiency in these dogs would make them a unique system in which to study chronic dermatomycosis, as a model for the disease in soldiers. It would also preclude misinterpretation of other studies (e.g., they might not reveal the potential of a test compound to provoke hypersensitization). Immune deficiency was suspected because they were extremely susceptible to bacterial infection, and necropsied newborns that died had a very small thymus gland.

#### RESULTS AND DISCUSSION OF RESULTS

Four punch-biopsy skin grafts were placed along the dorsal midline of each of 4 dogs: 2 Mexican hairless, another one with hair, and one dog of another breed. Each received a biopsy from each of the other dogs and one autograft.

All grafts were unsuccessful on the first attempt, the result of surgical failures after 10 days rather than immunologic rejection (even the autografts failed). The procedure was repeated with the same negative results.

#### CONCLUSIONS

We have more to learn about canine skin graft techniques.

#### RECOMMENDATIONS

The circumstantial evidence for immune deficiency is strong and certain knowledge of it would be useful. Persons with the necessary specialized skills should be engaged to help evaluate these unusual dogs.

#### PUBLICATIONS

None

STUDY NO. 5

Physiological and biochemical  
characteristics of domestic swine used  
in military research

#### PROBLEM

Mongrel dogs have served as the predominant large animal species for

medical research. Such usage is attributable to tradition and the ready availability of dogs at local pounds and animal shelters. In recent years, however, the use of dogs in medical research has come under increasing criticism from antivivisectionists in the general populace and from the scientific professions. Criticism by antivivisectionists is mostly based on ethical or emotional grounds; the predominant theme is the unnecessary suffering or mutilation of pets in research projects of dubious merit. Criticism by scientists falls into two categories. One criticism concerns uncertainties about the age, the genetic, nutritional, and environmental background, and the disease characteristics of mongrel animals acquired from dealers; the other criticism concerns the applicability to humans of data acquired in experiments on dogs because in many instances, canine response characteristics are vastly different from those observed in man.

The domestic pig offers an attractive alternative to the dog as a large animal model for human-oriented research. Its use has not elicited an emotional response from antivivisectionists, probably because swine are commonly slaughtered for meat and are not kept as life-long pets. Domestic pigs are readily available in all parts of the country. They are usually healthy and free of disease. They can be acquired in a variety of ages, sizes, and genetic backgrounds. Cost of acquisition and maintenance is usually far less than that of a dog of comparable size. A more important fact than these considerations exists, i.e., available information shows the pig is superior to the dog in terms of his physiological and biochemical similarities to man. Such information, however, is limited. Thus, if the pig is to become firmly established as a more appropriate large animal model than the dog for gathering experimental data which are applicable to humans, much additional research is needed to establish baseline data. It is to this problem that the present study is directed.

Two types of experiments are being conducted. In one, selected normal physiological or biochemical characteristics will be described. In the other, normal response characteristics of selected physiological or biochemical variables will be delineated.

#### RESULTS AND DISCUSSION OF RESULTS

The blood gas, acid-base status, and electrolyte levels of arterial blood were measured in 15 young domestic pigs of both sexes under control conditions which simulated those of combat casualties undergoing thoracic surgery. Ventilation inspired gas concentrations and anesthesia were regulated to maintain an arterial pH of about 7.40 and a  $P_{O_2}$  of about 100 mm Hg for at least 30 minutes before blood samples were obtained. Seven sites (femoral artery, femoral vein, posterior vena cava, anterior vena cava, pulmonary artery, internal jugular vein, and coronary sinus) were sampled. Although the resultant data are still being reduced and evaluated statistically, it appears that the biochemical characteristics of porcine blood in most instances are similar to those of human blood.

Selected mean values, compared to those commonly seen in humans and dogs, are as follows:

<u>Component</u>	<u>Swine</u>	<u>Human</u>	<u>Dogs</u>
Total Serum Protein (g/dl)	6.6	6.9	6.3
Serum Albumin (g/dl)	4.0	4.0	3.0
Serum Globulin (g/dl)	2.6	2.9	3.3
A/G Ratio	1.53	1.38	0.91
Arterial $\text{PCO}_2$ (mmHg)	47.0	40.0	32.8
Arterial $\text{HCO}_3^-$ (mEq/l)	27.6	24.5	21.3
Arterial $\text{Na}^+$ (mEq/l)	138	139	147
Arterial $\text{Cl}^-$ (mEq/l)	100	102	114

#### CONCLUSIONS

The blood gas and acid-base status of anesthetized pigs more closely approximates that of humans than does the blood gas and acid-base status of dogs.

#### RECOMMENDATIONS

1. Data reduction and statistical evaluations of porcine blood gas and acid-base characteristics should be completed.
2. These characteristics should be compared to those of dogs and humans.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD FORM 1498 (AK) 10 76	
3. DATE PREV SUMMARY <sup>a</sup>	4. KIND OF SUMMARY <sup>a</sup>	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA: CONTRACTOR ACCESS <sup>a</sup> <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
77 07 19	D. CHANGE	U	U	NA	NL		
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62172A	3M162172A812	00	003			
B. KKKKKKKKK	62772A	3M762772A812	00	003			
C. KKKKKKKKK	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Improvement of Health and Effectiveness of Military Dogs							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002600 Biology; 012900 Physiology; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-house	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING			
B. NUMBER <sup>a</sup>				FISCAL		77	
C. TYPE: Not Applicable				YEAR		1.0	
D. KIND OF AWARD:				CURRENT		56	
E. CUM. AMT.				78		1.3	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> : Letterman Army Institute of Research				NAME <sup>a</sup> : Letterman Army Institute of Research			
ADDRESS <sup>a</sup> : Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> : Department of Comparative Medicine Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> : Hannon, J.P., DAC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-4004			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Bucci, T.J., LTC, VC			
				NAME: POC: DA			
22. KEYWORDS (Precede Each with Security Classification Code) <sup>a</sup> (U) Physiology; (U) Nutrition; (U) Bioenergetics; (U) Combat Patrols; (U) Metabolism; (U) Endurance; (U) Biological Detectors Performance							
23. TECHNICAL OBJECTIVE <sup>a</sup> , 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Dogs are used extensively by US Armed Forces in conjunction with combat operations, as sentries for important installations in CONUS and OCONUS, as sensitive detectors of explosives and contraband, and for many other duties which cannot be performed reliably by any other detector system. A need exists for detailed information about how endurance may be improved by training, diet, or other procedures. Laboratory and field tests will be developed to evaluate nutritional and physiologic aspects of performance. Means will be sought to identify and ameliorate or eliminate chronic diseases which impair the effectiveness of trained dogs. 24. (U) Alterations in bioenergetics, metabolism, nutritional status and cardiopulmonary functions will be studied in military working dogs during exercise and training. Food requirements and body composition changes associated with specific levels of physical activity will be delineated. Factors impairing physical performance, such as high temperature and humidity, will be investigated. Diseases which impair effectiveness will be characterized by advanced laboratory means. 25. (U) 7610-7709 After a nine-month delay in delivery, four treadmills have been installed and calibrated, and eight German Shepherd dogs are being trained to exercise on them. Data collected during FY 78 will form the basis for understanding the nutritional bioenergetics of the military working dog. A radioisotopic bone scan technique was more sensitive than conventional radiography in detection of bone lesions of canine panosteitis. The scan will be a useful research tool in further study of this debilitating disease of German Shepherd dogs.							

# ABSTRACT

PROJECT NO.	3M762772A812	Military Animal Resources Development
WORK UNIT NO.	003	Improvement of Health and Effective- ness of Military Dogs

The following investigation has been conducted under this work unit:

STUDY NO. 1 The bioenergetics of exercise and physical training in  
the German Shepherd

Initiation of this study has been hampered by extended delays in the procurement and installation of animal treadmills to be used in the physical training of German Shepherd dogs. Additional delays resulted from the poor exercise tolerance of laboratory reared animals. Both of these problems have been resolved and experiments to determine the energy costs and biochemical characteristics of physical training are currently in progress.

## BODY OF REPORT

WORK UNIT NO. 003

Improvement of Health and Effectiveness  
of Military Dogs

STUDY NO. 1

The bioenergetics of exercise and  
physical training in the German  
Shepherd

### PROBLEM

The German Shepherd dog, because of his size, intelligence, and adaptability to physical and obedience training, has been extensively used for military patrol and sentry duties. Field reports, however, indicate that substantial numbers of military working dogs did not perform at anticipated levels during the Vietnam conflict. The factors responsible for such performance decrements are unknown, but speculative causes include an inadequate caloric intake and poor tolerance to hot, humid environments. The objective of this study is to delineate the physiological, biochemical, and nutritional factors which limit physical performance of the German Shepherd dog.

### RESULTS AND DISCUSSION OF RESULTS

Initiation of this study has been hampered by 2 major problems. First, the delivery and installation of canine treadmills was delayed for 9 months. Thus, actual physical training of animals did not commence until March 1977. Second, German Shepherd dogs derived from the Biosensor program but born and reared under laboratory conditions were found to be unacceptable subjects for a rigorous physical training program. They showed either poor adaptability to treadmill running or recurrent lameness. In many, but not all instances, the latter was associated with hip dysplasia of varying degree. Because of these problems with laboratory reared animals, German Shepherd dogs were procured from a licensed animal dealer. This required several months since acceptable animals (in terms of age similarity and absence of hip dysplasia) were not readily available. Eventually, 8 animals were procured and all were found to be adaptable to a treadmill-based training program. Initial experimental work, directed at the energy cost and biochemical characteristics of physical training, is currently underway.

### CONCLUSIONS

None

### RECOMMENDATIONS

1. The initial experiments should be completed.



2. Experiments to define the heat and humidity tolerance levels of exercising German Shepherd dogs should be initiated.

#### PUBLICATIONS

1. RODKEY, W.G., J.P. HANNON, J. DRAMISE, R.D. WHITE, D.C. WELSH, and B.N. PERSKY. Arterial capillary blood used to determine the acid base and blood gas status of dogs. Am J Vet Res 39:459-464, 1978
2. TURNIER, J.C., and S. SILVERMAN. Panosteitis: Comparison of radiographic and radioisotopic studies. J Am Vet Med Ass (in press)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD DR&E(AR)1636	
3. DATE PREV. SUMM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DDB'S INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM <sup>a</sup>
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3S162172A814	00	015			
b. <del>SECONDARY</del>	62772A	3M762772A812	00	004			
c. <del>THIRDARY</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Animal Models for Surgical Repair of Musculoskeletal Structures							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology; 003500 Clinical Medicine; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 05		CONT		DA		C. In-house	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
c. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		2.5	
d. TYPE				CURRENT		42	
e. KIND OF AWARD:				78		2.5	
f. CUM. AMT.						42	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Geasling, Jay W., CPT, VC			
				NAME: Lollini, Lance O., MAJ, MC POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Surgical Repair; (U) Extensor Tendon; (U) Nerve; (U) Muscle Transplantation; (U) Trauma; (U) Nerve Graft; (U) Microsurgical Technique							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Disabling injuries to the extremities of military personnel have proven extremely costly. To minimize lost duty days, permanent patient disability, and the expenditure of medical resources which result from such injuries, efforts are being made to refine and advance the current surgical techniques used in the treatment of wounds to the extremities. Through these studies, the objective is to provide data for the optimal repair and management of such injuries to return personnel to duty with maximum function in the minimum time.							
24. (U) The traumatized ulnar nerves of cats which had segmental loss were repaired either with interfascicular grafts or by an epineurial technique under tension. Six months after repair, critical evaluations of the neurorrhaphies for motor function, muscle efficiency, and total axon regrowth are made. Severed extensor tendons in rhesus monkey hands were repaired and immobilized for various periods of time to determine optimum immobilization and to evaluate the processes of extensor tendon healing. Numerous techniques have been used to freegraft partial or entire skeletal muscles in cats. The autotransplanted muscles have been evaluated by electromyography.							
25. (U) 76 10 - 77 09 Initial data indicate that an epineurial neurorrhaphy under tension, when immobilized three weeks postoperatively, provides as satisfactory a return of function as multiple interfascicular grafts in the cat. Total axon counts are now being tabulated; this should be the most important information. Although tenodesis of extensor tendons did not occur even after 70 days of immobilization following repair, preliminary data suggest the optimal period of immobilization is 18 to 24 days. Attempts to freegraft an entire muscle have been unsuccessful, but if minced, the muscles appeared to grow new fibers and definitely had electrical activity.							

<sup>a</sup> Available to contractors upon originator's approval

# ABSTRACT

PROJECT NO. 3M762772A812 Military Research Animal Resources  
WORK UNIT NO. 004 Animal Models for Surgical Repair of  
Musculoskeletal Structures

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 Nerve repair in cats: grafts vs tension

STUDY NO. 2 Digital extensor tendon repairs in monkeys

STUDY NO. 3 Study of autogenous graft techniques to restore  
muscle functions after traumatic injury

STUDY NO. 1. The ulnar nerve of the domestic cat was used as a model for repair of lacerated peripheral nerves. Sixteen animals underwent bilateral ulnar neurorrhaphy after a 2 cm segment of the nerve was removed. One side was repaired by the epineurial technique under tension, and the other side was repaired by using multiple nerve grafts. All cats were evaluated for return of function 6 months following the nerve repairs. From the data analyzed, there is no statistical difference between the two repair techniques.

STUDY NO. 2. Twenty-four adolescent rhesus monkeys underwent common digital extensor tendon transection and repair in the mid metacarpal region of the index and little fingers. Sequential biopsies elicited the healing process involved, and various periods of immobilization resulted in no significant differences in postoperative complications. Tensile strength determinations showed a gradual increase in the strength after the 21st postoperative day unrelated to the length of immobilization.

STUDY NO. 3. Several methods have been used to autotransplant partial or entire skeletal muscles in cats. Some of the grafts have been "preconditioned" by denervation 2 to 3 weeks prior to transplantation, but others have been grafted without prior denervation. Some sections have been minced at the time of transplantation. Vascular flaps and microvascular anastomoses have not yet been employed. Electromyographic studies have been done on all of the grafted muscles with the regrown minced muscle sections maintaining voluntary functions for the greatest period of time.



## BODY OF REPORT

WORK UNIT NO. 004

Animal Models for Surgical Repair of  
Musculoskeletal Structures

STUDY NO. 1

Nerve repair in cats: grafts vs tension

### PROBLEM

Peripheral nerve injuries are common in both combat and noncombat military accidents. Many of the war injuries from the Vietnam conflict included severe damage to the peripheral nerves of the upper and lower extremities. During one 24 month period, 54% of all casualties in military hospitals had such injuries. Although our technical capabilities have progressed greatly during the last several years in the surgical repair of peripheral nerves, we still do not have a good method of managing segmental nerve defects. Tension at the repair site is considered detrimental to nerve regeneration and healing. Consequently, the use of a multiple nerve graft has been advocated. Problems of repairing a nerve under tension, where joints must be flexed, nerves must be mobilized, and vascularity diminished, are not completely overcome by the use of multiple nerve grafting procedures in which an avascular unmatched segment is used to bridge the defect and relieve tension. Intrafascicular grafting not only results in the interposition of an avascular segment which loses all endoneurial elements and structure, but this technique also requires two separate neurorrhaphies which regenerating neurites must cross. We know of no evaluation comparing nerve repairs with tension to those neurorrhaphies which have been done with the use of multiple grafts. This study critically compares, by objective evaluation, epineurial end-to-end repairs with tension to interfascicular grafts done without tension following loss of a nerve segment.

### RESULTS AND DISCUSSION OF RESULTS

We have previously described an experimental model for peripheral nerve repair by using both ulnar nerves of domestic cats. In this study, 16 house cats underwent bilateral resection of a 2 cm length of the ulnar nerve above the medial humeral epicondyle. One nerve was sutured under tension with size 8-0 nylon by using an epineurial technique. The other nerve was repaired by using multiple caudal cutaneous sural grafts that eliminated all tension at both suture lines. Size 10-0 nylon was used to suture the grafts. The cats were evaluated for comparison of return of function 6 months after the nerve sutures. Subjective evaluation included observation of gait, ability to fan claws (intrinsic function), and withdrawal from pin prick (sensation). Objective evaluation included efficiency and maximum strength of the ulnar innervated flexor muscles, weight of the flexor carpi ulnaris muscle, and regrowth of myelinated nerve fibers by total axon counts proximal and distal to the repairs. Histochemical stains of the

flexor carpi ulnaris muscle were done to determine fiber types and grouping. The axon counts and evaluation of histochemical stains are currently in progress. All other evaluations have been completed and statistically analyzed. Statistical analysis of data showed no significant differences between the two techniques in overcoming segmental nerve defects in cats. When compared to an initial study, where nerves were repaired primarily without tension, it was found that all animals with segmental defects had less return of function than those animals which had no segmental defect but merely an acute laceration and repair.

#### CONCLUSIONS

We do not yet have the answer to the optimal management of segmental defects of peripheral nerves. Since we have demonstrated a qualitative and quantitative difference between the results of nerves repaired without tension, either epineurially or perineurially, when compared to nerves with segmental defects repaired under tension, or with grafts, we concluded that the ideal neurorrhaphy is one performed as soon after the injury as possible, without tension, without grafts, with atraumatic technique, with appropriate alignment of fascicular nerve ends, and with the realization by the surgeon and the patient that perfect functional return is not possible.

#### RECOMMENDATIONS

To confirm the validity of our findings, we feel further extensive evaluations of peripheral nerve repairs should be conducted in nonhuman primates. Attempts are underway and should continue to identify and describe a method of counting the axons by computer or other electronic means, greatly speeding future studies and adding to the overall validity. Clinical correlation of Letterman Army Medical Center patients with peripheral nerve injuries should be coordinated with prospective primate studies.

#### PUBLICATIONS

1. CABAUD, H.E., W.G. RODKEY, H.R. MC CARROLL, Jr., S.B. MUTZ, and J.J. NIEBAUER. Epineurial and perineurial fascicular nerve repairs: A critical comparison. J Hand Surg 1:131, 1976

STUDY NO.           2                           Digital extensor tendon repairs in monkeys

#### PROBLEM

Study of tendon injuries and their repair has centered around flexor tendons. Lack of attention to extensor tendon injuries may be attributed to the fact that extensor tendons are the positioners of the hand and fingers, allowing the flexors to perform the primary functions of pinch, grasp, and hook. Consequently, there is a paucity

of published data regarding healing of extensor tendons, even though extensor tendon injuries are common in both combat and noncombat situations. When dealing with a clean, sharp transection of an extensor tendon, it is general practice to reapproximate the severed ends surgically and immobilize the injured part during healing. The optimum duration of immobilization, however, is subject to great controversy. Prolonged immobilization of a repaired extensor tendon may result in increased scar formation, tenodesis, and contracture. This may result in a temporary or permanent disability of the part served by the tendon, and additional surgical procedures such as tenolysis may be required to restore mobility to the tendon. Conversely, too early use of a repaired extensor tendon may result in repair failure. Again, this leads to additional surgical procedures to correct the problems. This study has been designed to determine the optimal period of immobilization of a repaired extensor tendon, and to help identify and describe the cellular processes involved in healing of extensor tendons repaired after transection.

#### RESULTS AND DISCUSSION OF RESULTS

Experiment 1. The common digital extensor tendon of the index and little fingers of one hand of 9 rhesus monkeys were completely severed and surgically reunited by standard techniques. The arm and hand were then immobilized in a full arm cast with the elbow in flexion. Immobilization for each monkey was either 2, 4, 7, 10, 14, 21, 28, 35, 42, or 70 days, the period assigned randomly. At the appropriate time, biopsy specimens of each repaired tendon were removed. The index finger biopsies were used for histopathologic studies, and the small finger biopsies were used to determine tensile strength of the repair with the use of a tensiometer. Histologically, the tendon appears to heal in 2 ways. First, granulation from the surrounding injured tissue forms a sheath around the injured segment which gradually matures and organizes and fills any defects between the ends of the severed tendon. Vascularity decreases within the regenerating tendon and the extracellular collagen coalesces. Second, fibroblast nuclei within the injured tendon also appear to proliferate. Although no mitotic figures were observed, rows of plump nuclei were widely separated from the granulation sheath. The tensile strength determinations indicate that the tendon does not begin to develop appreciable strength until after postoperative day 21. This indicates that limited movement after such time can be tolerated, and before this time there is no appreciable difference from the first postoperative day.

Experiment 2. Goniometric measurements to establish range of motion of the metacarpophalangeal (MCP), proximal (PIP) and distal (DIP) interphalangeal joints were performed on one hand of 15 rhesus monkeys immediately prior to surgery. A surgical procedure identical to that of Experiment 1 of the study was performed. Operated hands were in a cast for 2, 3, or 4 weeks of total immobilization, followed in each case by 2 weeks of light activity in a bulky dressing. At the



appropriate time after immobilization, animals were evaluated by goniometric measurements for range of motion of the MCP, PIP, and DIP joints of the index and small fingers. Measurements were also repeated at 6, 8, and 10 weeks after surgery. Differences as a function of duration of immobilization were sought. The group immobilized totally for 4 weeks showed decreased passive motion when goniometrically measured at 42 days when compared to the other 2 groups. By 70 days, all groups were equal and nearly all of the goniometric measurements had returned to normal preoperative values.

#### CONCLUSIONS

We have adequately described the histologic appearance of the healing extensor tendon. We have also found that the healing extensor tendon of the rhesus monkey does not develop appreciable strength until after 21 days following surgery. However, our model has not developed 2 of the major problems we intended to evaluate, i.e., tenodesis or extensor lag. Either treatment groups are all equally suitable for the management of the injury we produced, or the model is inappropriate to evaluate these problems.

#### RECOMMENDATIONS

All monkeys used in this study were equivalent in age to human adolescents. This young age may partially explain the excellent healing found in all treatment groups. We feel, therefore, that Experiment 2 should be repeated in a small number of older, more mature animals to determine if tenodesis develops more readily in these older monkeys. Such determinations will further confirm the adequacy of our model.

#### PUBLICATIONS

None

STUDY NO. 3

Study of autogenous graft techniques to restore muscle functions after traumatic injury

#### PROBLEM

Limb and skeletal muscle viability may be impaired or often destroyed following severe trauma or limb swelling and compartmental syndrome. Subsequent surgical treatment, which out of necessity includes extensive debridement of all devitalized tissues, further adds to the functional loss and disfigurement of the affected part. Currently, there is no technique to restore or augment severely traumatized skeletal muscles following such functional loss. Small muscles have been used successfully to restore function to sphincters and small areas of the face in human patients, but there are no reports of successfully replacing large muscle masses. This study is designed to develop

techniques in animal models which will permit large muscle masses to be autotransplanted to another area of the same limb or a different limb, restoring function to a severely traumatized part.

#### RESULTS AND DISCUSSION OF RESULTS

Numerous varied techniques have been used to free graft skeletal muscles in cats. Attempts have been made to graft autogenously both partial and whole muscles. Initially, the muscles were preconditioned by denervating them 2 to 3 weeks prior to autotransplantation. When compared to those muscle grafts which had not been preconditioned, it was found that the preconditioned muscle grafts were far inferior. Electromyographically, several of the nonpreconditioned muscle grafts have shown voluntary contraction and strong electrical activity for up to 12 weeks following autotransplantation. The preconditioned muscle grafts universally have failed to exhibit any electrical activity or motor unit potentials at all following autotransplantation. Those grafts which remained viable in activity for at least 12 weeks progressively scarred and fibrosed until nothing but scar tissue remained. Based on this observation, it was concluded that the grafts were not receiving enough surface exposure to the host muscle bed to gain adequate vascularization to support and maintain viability. Consequently, some grafts were minced mechanically prior to their autotransplantation, and these were found histologically to be growing new muscle fibers, and also appeared to have viable motor end plates. Another technique has now been tried to move the whole intact muscle and provide more surface exposure to the receptor muscle bed.

These grafts have recently been biopsied, and we are awaiting the results of the histochemical and histologic examinations. Autotransplants in which microneurovascular anastomoses are utilized have not yet been done.

#### CONCLUSIONS

We believe that preconditioning a muscle graft by prior denervation not only is unnecessary, but it appears to be detrimental to the viability of the graft. Large muscle masses can be autotransplanted as free grafts, and they will regain viability and some electrical activity. However, it seems that, as the metabolic activity of these muscles increases, the neovascularity is too small to maintain a functional muscle unit. Consequently, techniques must be developed to provide an increased blood supply to these free grafts, especially at the time when the metabolic and electrical activity starts to increase rapidly.

#### RECOMMENDATIONS

Efforts should continue to describe a technique which will permit large skeletal muscle masses to be moved to different areas of the same body

to help restore function to a damaged or devitalized limb. It may be necessary to accomplish this as a multiple stage procedure so that each of the various stages will be able to undergo complete neovascularization prior to the addition of the next graft. In addition to the electromyographic examination of these muscle grafts, attempts should be made to measure the actual contractility of the grafts. Microsurgical neurovascular free-muscle grafts should be evaluated and compared to free-muscle grafts.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISB <sup>a</sup> INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
77 07 19	D CHANGE	U	U	NA	NL		
10. NO./CODES <sup>a</sup>		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62172A		3M162172A812		00 024	
b. CONTINUING TASK		62772A		3S762772A814		00 024	
c. CONTINUING TASK		CARDS 114f					
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Neurobehavioral Investigations of Military Trauma							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003800 Life Support; 012900 Physiology; 013400 Psychology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10 01		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING			
b. NUMBER <sup>a</sup>				FISCAL YEAR		2.5	
c. TYPE: Not Applicable				CURRENT		33	
d. AMOUNT:				78		3	
e. KIND OF AWARD:				68			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Pribyl, V. J., DAC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Combat Trauma; (U) Massive Transfusion;							
(U) Neurophysiology; (U) Resuscitation; (U) Psychopharmacology							
23. TECHNICAL OBJECTIVE <sup>a</sup> , 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Resuscitation from trauma in combat operations imposes problems not typically encountered in civilian medical operations. Among those are the occurrences of mass casualties, and physical limitations of field medical facilities. Short, violent conflicts will further limit field medical support of combat operations and medical casualties may be required to continue providing limited support of combat operations. There will be greater emphasis on methods of treating combat injuries which permit rapid return to duty. The objectives of this work unit are to assess the effects of military trauma on combat effectiveness and to evaluate functional recovery following the use of different resuscitating procedures.</p> <p>24. (U) The methods used emphasize the detection and quantification of those functional changes which could limit adaptation to the environment following trauma. Both behavioral and neurophysiological techniques will be employed in order to increase the chances of detecting effects which significantly impair the function of the organism. Behavioral testing will be used to evaluate basic sensory and motor processes as well as more complex cognitive processes. The additional data obtained through appropriate analysis of spontaneous and evoked electroencephalographic activity will provide further evidence of possible changes in central nervous system activity.</p> <p>25. (U) 76 10 - 77 09 Behavioral testing has been used to demonstrate significant differential effects of massive transfusion with various resuscitating solutions. Further studies will be required to obtain dose-response data for these materials, and to explain their behavioral effects.</p>							

<sup>a</sup> Available to contractors upon originator's approval

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

# ABSTRACT

PROJECT NO.            3M162172A812            Military Research Animal Resources  
TASK NO.  
WORK UNIT NO.        024                    Neurobehavioral Investigations  
   of Military Trauma

The following investigations have been conducted under this work unit:

STUDY NO. 1 Behavioral effects of massive transfusion

STUDY NO. 2 Neurophysiological effects of massive transfusion

STUDY NO. 3 Servoanesthesia

STUDY NO. 1. Operant behavior of rats was examined following exchange transfusion with asanguinous solutions. Different transient performance decrements were observed following transfusion with albumin, stroma-free hemoglobin, or a mixture of these proteins. Behavioral recovery appeared to be complete in all transfused groups by the second week of testing. Tissue samples obtained several weeks after transfusion revealed no morphologic differences between groups. Further studies will be required to determine the mechanisms responsible for the behavioral changes observed during these preliminary experiments.

STUDY NO. 2. Spontaneous and stimulus-evoked brain electrical activity was recorded from chronically implanted electrodes. Digital signal processing was used to obtain averaged event-related potentials and to obtain power spectra of electroencephalographic signals. When sufficient baseline data are obtained, similar techniques will be used to study brain electrical activity during and after massive transfusion. Data obtained during these experiments will be related to behavioral measures of functional changes in the central nervous system.

STUDY NO. 3. Electroencephalographic activity (EEG) was recorded from animals anesthetized with halothane, methoxyflurane, or pentobarbital. Characteristic changes in patterns of electrophysiological activity were related to depth of anesthesia. Work is in progress to identify those features of the EEG which best discriminate levels of anesthesia. The reduced data set consisting of these selected features of the EEG will be used to regulate anesthesia delivery.

## BODY OF REPORT

WORK UNIT NO.	024	Neurobehavioral Investigations of Military Trauma
STUDY NO.	1	Behavioral effects of massive transfusion

### PROBLEM

The extreme conditions of combat operations pose difficulties in the supply and storage of whole blood. In an attempt to ameliorate these difficulties, research is in progress to solve problems associated with the long-term storage of blood and to develop new asanguinous resuscitating materials which may offer advantages in the front-line medical support of military operations. A research program has also been designed to test for brain dysfunction and/or brain damage following massive replacement of blood with standard or experimental resuscitating solutions. The purpose of this study was to use readily quantified measures of behavior obtained under controlled conditions to evaluate functional recovery of the central nervous system following massive transfusion with cell-free resuscitating solutions.

### RESULTS AND DISCUSSION OF RESULTS

The methods of behavioral evaluation were based upon operant techniques in which rats were required to perform specific tasks in order to obtain food reinforcement. Two operant schedules were selected for preliminary study. A fixed ratio (FR) schedule provided reinforcement in direct proportion (1:20) to the number of bar-press responses emitted by the animal. The FR performance is characterized by relatively high and stable response rates. A differential reinforcement of low response rates (DRL) schedule required that subjects develop precise temporal spacing of responses in order to obtain food reinforcement. The DRL task was selected as a means of determining whether or not relatively complex timing behavior is preserved following massive exchange transfusion.

The FR and DRL operant behavior of rats was examined following 65 percent exchange transfusion with bovine serum albumin (BSA), stroma-free hemoglobin (SFH), or a mixture of these materials (MIX). The FR rates of transfused animals were significantly depressed in comparison to surgical controls 24 hr after exchange transfusion, but differences between transfused groups were not significant. After the first day, there were no significant differences in FR response rates between the surgical control, hemoglobin-albumin, and albumin groups. Response rates of the SFH group, however, remained significantly lower in comparison to all other groups through the fourth day of testing. Transfusion related changes in DRL performance were similar to those observed



with the FR task. Behavioral recovery appeared to be complete in all animals by the second week of testing. Behavioral testing was continued for six weeks. Animals were then sacrificed and tissue samples were obtained for histopathologic study. Light microscopic examination of eye, brain, lung, thymus, heart, liver, spleen, pancreas, kidney, mesenteric lymph node, and bone marrow revealed no morphologic differences between control, MIX, BSA, and SFH groups.

#### CONCLUSIONS

Significant changes in operant behavior were related to the type of material used for exchange transfusion. The apparently poorer performance of the SFH group must be interpreted with caution for the following reasons. First, SFH was nearly completely excreted at the time of initial behavioral testing. Second, the performance of the MIX group which also received hemoglobin was virtually identical to the group transfused with BSA alone. Finally, the stroma-free hemoglobin solution which was used was an experimental formulation and changes in the methods of preparation or administration might result in different behavioral effects. Therefore, it would be desirable to reevaluate SFH solutions as new variations are made available.

#### RECOMMENDATIONS

Studies of the behavioral consequences of massive transfusion should be continued with the use of standard crystalloids, colloids, and banked whole blood. Future research should include dose-response studies in which changes in behavioral indices will be related to amounts of blood replaced. Those studies should use blood replacement following hemorrhage in addition to the exchange transfusion procedure. The hemorrhage model is a more realistic representation of blood loss following trauma. Finally, behavioral testing should be extended to include evaluation of both more elementary and more complex forms of behavior following transfusion.

#### PUBLICATIONS

1. O'MARA, P.A., F. DEVENUTO, P.W. MELICK, R.A. PATTERSON, and G.E. ESGANDARIAN. Behavioral Effects of Massive Transfusion with Cell-free Resuscitating Solutions. Report No. 45. San Francisco, California: Letterman Army Institute of Research, January 1978
2. O'MARA, P.A., F. DEVENUTO, and G.E. ESGANDARIAN. Fixed Ratio Performance in Rats Following Massive Transfusion with Cell-free Resuscitating Solutions. Report No. 46. San Francisco, California: Letterman Army Institute of Research, January 1978

PROBLEM

Examination of stimulus-evoked peripheral and central nervous system electrophysiological events can provide valuable information concerning the ability of the nervous system to process and respond to external events. Changes in the spontaneously occurring electrical activity of the brain may provide additional information concerning the general state of the organism. The objectives of this study are (1) to use electrophysiological data in assessing the effects of various resuscitating solutions and procedures on brain functioning and behavior and (2) to relate electrophysiological data obtained during resuscitation to the subsequent functional recovery of the subject. Suspected post-traumatic functional changes in the subjects will also be confirmed with behavioral measurements.

The identification of physiological variables which reliably predict overall functional recovery could lead to the development of diagnostic procedures for use in rapid screening of brain dysfunction in combat casualties where significant blood loss and replacement have occurred.

RESULTS AND DISCUSSION OF RESULTS

Baseline visual and somatosensory-evoked potential data have been recorded from rats with the use of chronically implanted electrodes. Digital computer programs were developed which allow on-line monitoring of spontaneous and stimulus-evoked electroencephalographic activity during resuscitation procedures and during recovery. Investigations are now in progress to assess changes in brain electrical activity during hemodilution with standard asanguinous resuscitating solutions. In future studies, electrophysiological data recorded during resuscitation will be related to behavioral recovery in the same subject.

CONCLUSIONS

None

RECOMMENDATIONS

Statistical analyses of data from the initial experiments must be completed.

PUBLICATIONS

None

PROBLEM

Several anesthetics produce characteristic changes in the spontaneous electrical activity of the brain, and the changes can be correlated with the depth of anesthesia. It would appear feasible, therefore, to use selected features of the electroencephalogram to control automatically the delivery rate of anesthetic agents and consequently maintain a given depth of anesthesia. Historically, attempts to implement servoanesthesia systems have met with limited success due to the complexities of biological signal processing and the construction of the control systems for anesthesia delivery. There were also unresolved questions concerning the selection of appropriate features of the biological signals for use in feedback regulation. Recent developments in digital signal processing offer practical solutions to these difficulties. This study was undertaken in order to re-examine the servoanesthesia problem by using the more advanced computer-assisted methods which are currently available. Servoanesthesia would be particularly valuable in forward medical support of combat operations.

RESULTS AND DISCUSSION OF RESULTS

Spontaneous and stimulus-evoked electroencephalographic (EEG) activity was recorded from animals anesthetized with halothane, methoxyflurane, or pentobarbital. Other physiological variables including body temperature and heart rate were also monitored. Spectrum analyses and signal averaging techniques were used to reduce EEG data. Preliminary data analyses confirmed the presence of previously reported EEG changes related to depth of anesthesia. Further statistical analyses will be required to evaluate the stability of the observed EEG changes as a function of time (duration of anesthesia) and to identify features of the reduced data set which best discriminate levels of anesthesia. Work is now in progress to develop computer programs which can be used for on-line processing of the physiological data for use in feedback control of an anesthetic delivery system.

CONCLUSIONS

None

RECOMMENDATIONS

Data collection and statistical analyses should be continued.

PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6079	77 10 01	DD DR&E(AR)6.16	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DES'N INSTR <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
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11. NO. / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62172A	3E162172A813		00	021		
b. <del>XXXXXXXXXX</del>	62772A	3E762772A813		00	021		
c. <del>XXXXXXXXXX</del>	CARDS 114f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Determination of Threshold Data from Coherent and Incoherent Radiation Sources							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
09600 Masers and Lasers; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 12		Cont		CA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER <sup>a</sup>				77		3.5	
c. TYPE: Not Applicable				FISCAL YEAR		65	
d. AMOUNT:				CURRENT		78	
e. KIND OF AWARD:				5.5		130	
f. COM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> : Letterman Army Institute of Research				NAME <sup>a</sup> : Letterman Army Institute of Research			
ADDRESS <sup>a</sup> : Presidio of San Francisco, CA 94129				Division of Non-Ionizing Radiation			
				ADDRESS <sup>a</sup> : Department of Biomedical Stress			
				Presidio of San Francisco			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-2732			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Stuck, B. E., DAC			
				NAME:			
				POC DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Eye Protection; (U) Infrared Lasers							
(U) Systems Safety; (U) Laser Hazard; (U) Eye Damage; (U) Skin Damage							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objectives are to experimentally determine dose response relationships for exposure to infrared laser radiation for exposure conditions relevant to DARCOM laser systems operation, for systems used for ECOM designation and guidance tasks and to recommend to the Army user community permissible exposure limits and field safety procedures.							
24. (U) The ED <sub>50</sub> (effective dose required to produce a change 50% of the time) for various exposure conditions and response criteria were determined. The cornea exposures are evaluated at various time intervals by direct observation, histological techniques, and specular microscopy.							
25. (U) 7610-7709 An ED <sub>50</sub> of 2.9 joules/cm <sup>2</sup> was determined for a holmium laser operating at 2.06 microns. The multimode laser was operated in the long pulse mode with a pulse duration of 250 μsec. The intensity distribution was irregular and the beam diameter was 1.8 mm at the 1/e irradiance points. The corneal opacities involved the corneal epithelium and upper stroma. Persistent stroma scars were observed three months after exposure at doses 2.5 times the ED <sub>50</sub> . Routine histological evaluation showed changes in the basal epithelial cells for doses near the ED <sub>50</sub> . The ED <sub>50</sub> for full corneal exposure (beam diameter 7.4 mm at 1/3 irradiance points) has been estimated to be 17.5 watts/cm <sup>2</sup> laser radiation at 10.6 microns. Corneal specular microscopy has shown endothelial cell changes at 24 hours after the exposure for doses near the ED <sub>50</sub> .							

# ABSTRACT

PROJECT NO. 3E7672772A813

Health Effects of Military Lasers

WORK UNIT NO. 021

Determination of Threshold Data  
from Coherent and Incoherent  
Radiation Sources

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 Ocular and skin effects of infrared laser radiation

An  $ED_{50}$  of  $2.9 \text{ J/cm}^2$  was determined for a holmium laser operating at 2.06 microns. The multimode laser was operated in the long pulse mode with a pulse duration of 250  $\mu\text{sec}$ . The intensity distribution was irregular and the beam diameter was 1.8 mm at the  $1/e$  irradiance points. The corneal opacities involved the corneal epithelium and upper stroma. Persistent stroma scars have been observed three months after exposure at doses 2.5 times the  $ED_{50}$ . Routine histological evaluation showed changes in the basal epithelial cells for doses near the  $ED_{50}$ . The  $ED_{50}$  for full corneal exposure (beam diameter 7.4 mm at  $1/e$  irradiance points) has been estimated to be  $17.5 \text{ watts/cm}^2$ . Corneal specular microscopy has shown endothelial cell changes at 24 hours after the exposure for doses near the  $ED_{50}$ .

( $ED_{50}$  is defined as the probability of producing a clouding of the epithelium and/or superficial stroma in fifty percent of exposures one hour after laser exposure.)

## BODY OF REPORT

WORK UNIT NO.	021	Determination of Threshold Data from Coherent and Incoherent Radiation Sources
STUDY NO.	01	Ocular and skin effects of infrared laser radiation

### PROBLEM

Many present and proposed military laser systems operate in the infrared region of spectrum beyond 1.4 microns. This region of the electromagnetic spectrum is commonly referred to as the "eye safe" region because the radiant exposure required to produce an observable change is 10 to 10000 times higher than that required for visible and near infrared wavelengths. For infrared wavelengths, the cornea and outer ocular structures are the primary absorption site and consequently the site of injury whereas the retina is affected by the visible and near infrared wavelengths. Although permissible exposure limits have been defined in TB Med 279, there are exposure conditions relevant to military applications where no bioeffects data are available. Permissible exposure limits have been established by extrapolation for these conditions. The specific problems addressed by this study are to (1) determine dose-response relationships for holmium laser radiation at 2.06 microns, (2) evaluate epithelial and endothelial changes from CO<sub>2</sub> laser radiation at and above present permissible exposure limits, and (3) evaluate these bioeffects data with respect to present standards.

### RESULTS AND DISCUSSION OF RESULTS

A holmium laser (fabricated by Electronics Command, Ft. Monmouth, NJ) was used to determine the ED<sub>50</sub> (effective dose for the 0.50 probability of observing the response) for observation of a corneal opacity 1 to 2h after the exposure. The laser was operated in the long pulse mode with a pulse duration of 250  $\mu$ sec. The measured beam divergence of the laser was approximately 5.0 mrad. Because of the large divergence and limited energy output per pulse, a four diopter lens was used to focus the radiation on the cornea. At the corneal plane, the intensity distribution was bilobal with a 1/e diameter of 1.8 mm. The dose represented in this report is the estimated peak radiant exposure at the corneal plane. The cornea of seven rhesus monkeys were used in these experiments. Five exposures were placed in each eye. The dose was randomly varied from 1.0 to 6.0 J/cm<sup>2</sup>. The ED<sub>50</sub> for biomicroscopically observing a corneal lesion was 2.9 J/cm<sup>2</sup> with a 95% confidence interval about the ED<sub>50</sub> of 2.7 to 3.1 J/cm<sup>2</sup>. The slope of the dose response



curve was 1.17 ( $ED_{84}/E_{50}$ ) which is comparable to corneal dose-response curves for the  $CO_2$  laser wavelength of 10.6 microns. The lowest dose where a lesion was observed was  $2.4 \text{ J/cm}^2$ . Lesions near the threshold ( $2.7\text{--}3.1 \text{ J/cm}^2$ ) were not apparent by slit lamp observation one week after the exposure, however, for higher doses ( $3.4 \text{ J/cm}^2$ ) a stromal scar (the upper 1/3 of stroma) was still obvious 1 month after exposure. Preliminary light microscopy suggests that the locus of alteration is in the basal epithelial cells of the cornea.

Typical field exposure to  $CO_2$  laser radiation will involve the exposure of the entire cornea. Beam diameters used to determine dose response relationships for the corneal effects of  $CO_2$  laser radiation have been 2 to 3 mm at the 1/e intensity points. In this study, the cornea of 5 rhesus monkeys were exposed to various doses of  $CO_2$  laser radiation for a 100 msec exposure duration. The beam intensity distribution was Gaussian with the 1/e irradiance diameter of 7.4 mm. On the basis of 10 exposures with doses varying from  $4.0$  to  $30 \text{ W/cm}^2$  (peak irradiance), the estimated  $ED_{50}$  for observation of a corneal opacity 1-2 h after the exposure is  $17.5 \text{ W/cm}^2$ . The  $ED_{50}$  for a 215 mm irradiance diameter was previously determined to be  $25 \text{ W/cm}^2$ . Consequently, a lower irradiance was required to produce a corneal lesion for a 7.4 mm irradiance diameter.

The effects of these exposures on the cornea endothelium are being evaluated by in vivo corneal specular microscopy. Appearance of the corneal endothelium and endothelial cell size is being compared before and after exposure. For the exposures near opacity threshold ( $18\text{--}22 \text{ W/cm}^2$  for 100 msec, 7.4 mm beam), dark enlarged intracellular spaces were observed at irregular intervals beneath the exposure site. Some larger endothelial cells were observed several weeks post exposure, however, further evaluation of endothelial cell size with dose and time after exposure is required.

Various degrees of vitreal syneresis were observed in 28 of the 30 rhesus monkey eyes. The observed vitreal structures varied from fine strands randomly spaced throughout the vitreous to thick intertwining fibrous networks with some clumping of the collagenous condensate at the fiber junctions. Qualitatively, the degree of syneresis was slightly more extensive in the eight older mature males than in the seven younger animals. In all animals, a clear view of the fundus could be obtained with the ophthalmoscope. The vitreous structures may be one cause of variability in ocular dose-response relationships for exposure to laser radiation. The effect on retinal exposure experiments of the finer vitreal structure is considered minimal.

Persistent ultrastructural changes have recently been observed for low level Q-switched ruby laser exposure. The present permissible exposure standard (TB Med 279) was compared with the doses required for 3 exposure conditions where dose response relationships for the

ocular exposure of rhesus monkeys to a Q-switched laser have been previously reported. The exposure conditions were (1) production of an ophthalmoscopically visible lesion for a minimal retinal irradiance diameter ( $\approx 50$  microns), (2) the production of an ophthalmoscopically visible lesion for a 1000 micron retinal irradiance diameter, and (3) the production of prolonged electron microscopic changes in the outer segments of photoreceptors for a 1000 micron retinal irradiance diameter, and (3) the production of prolonged electron microscopic changes in the outer segments of photoreceptors for a 1000 micron retinal irradiance diameter. By using the intrabeam viewing standard, the dose required for the production of an ophthalmoscopically visible lesion in the macula for a minimal retinal irradiance diameter was 50 times greater than the MPE. The doses for large retinal irradiance conditions of 2 and 3 were compared to the extended source viewing standard by calculating the dose received from an extended source that is irradiated at the MPE and imaged to 1000 microns on the retina. This calculated total intraocular energy (TIE) for a maximally dilated pupil is less than 10 times below that required for condition 2 and approximately twice the dose used to produce electron microscopic changes in condition 3. If one is concerned about ultra-structural changes to the retina from a single Q-switched ruby laser pulse, the present standard for these specific conditions is certainly not too conservative.

#### CONCLUSIONS AND RECOMMENDATION

Further research is required for relevant wavelengths from 1.4 to 4.0 microns. The roll of the endothelium in cornea injury and repair must be evaluated for different infrared wavelengths and exposure conditions. These evaluations should be made for doses near permissible exposure limits. The effects of repetitive pulses for far infrared laser wavelengths need to be investigated. The permissible exposure limits must be evaluated with respect to current bioeffects research.

#### PUBLICATIONS

1. STUCK, B. E. Comparison of the Maximum Permissible Exposure with the Doses Required to Induce Retinal Alterations from Q-Switched Ruby Laser Exposures. Report No. 35. Presidio of San Francisco, California: Letterman Army Institute of Research, March 1977.
2. STUCK, B. E., D. M. TALSMA, and E. S. BEATRICE. Vitreal Syneresis of Rhesus Monkeys. Report No. 40. Presidio of San Francisco, California: Letterman Army Institute of Research, June 1977 (accepted for publication in Investigative Ophthalmology)
3. ZWICK, H., B. E. STUCK and D. JENKINS. Multiline Laser Protection The Copper Vapor Laser. Presidio of San Francisco, California: Letterman Army Institute of Research, May 1977.

4. BEATRICE, E. S., H. ZWICK, D. I. RANDOLPH, B. E. STUCK and D. J. LUND. Laser Hazards: Biomedical Threshold Level Investigations. Report No. 36. Presidio of San Francisco, California: Letterman Army Institute of Research, March 1977.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636																																									
3. DATE PREV SUMRY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISEM INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM																																								
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11. TITLE (Precede with Security Classification Code) <sup>a</sup>																																															
(U) System Developer Assistance Studies in Laser Bioeffects																																															
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>																																															
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION																																											
NAME <sup>a</sup> : Letterman Army Institute of Research				NAME <sup>a</sup> : Letterman Army Institute of Research																																											
ADDRESS <sup>a</sup> : Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> : Department of Biomedical Stress																																											
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TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-3344																																											
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:																																											
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS																																											
				NAME: Lund, D. J. DAC																																											
				NAME: POC:DA																																											
22. KEYWORDS (Precede EACH with Security Classification Code)																																															
(U) Ocular hazard; (U) Damage threshold (U) Laser Safety; (U) GaAs; (U) Neodymium;																																															
(U) Erbium; (U) Repetitive pulse																																															
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)																																															
23. (U) The objective is to provide data base for safety documentation of gallium arsenide laser training (GaAs) device MILES (Multiple integrated Laser Engagement System). TRADOC plans to employ several thousand MILES devices in combat training exercises within the next year.																																															
24. (U) By utilizing rhesus monkeys, we will determine ocular damage thresholds for neodymium laser pulse trains simulating the MILES code (approx. 3 kHz) and for erbium laser single pulse exposures, evaluate the retinal effects of a MILES prototype laser, and evaluate the retinal effects of a continuous wave GaAs laser.																																															
25. (U) 7610-7709 ED <sub>50</sub> doses (TIE for pulse train) were determined for neodymium laser pulse trains ( $\lambda$ -1060 nm, single pulse duration = 180 nsec, single pulse ED <sub>50</sub> =137 $\mu$ j TIE)																																															
<table border="0"> <thead> <tr> <th></th> <th>PRF</th> <th colspan="5">Number of Pulses</th> <th></th> </tr> <tr> <th></th> <th></th> <th>2</th> <th>3</th> <th>6</th> <th>74</th> <th>1000</th> <th></th> </tr> </thead> <tbody> <tr> <td>0.1 kHz</td> <td></td> <td>150</td> <td>270</td> <td></td> <td></td> <td></td> <td>TIE</td> </tr> <tr> <td>1.0 kHz</td> <td></td> <td>160</td> <td>153</td> <td>330</td> <td>1200</td> <td>10100</td> <td>(microjoules)</td> </tr> <tr> <td>3.0 kHz</td> <td></td> <td>121</td> <td>131</td> <td>182</td> <td></td> <td></td> <td>)</td> </tr> </tbody> </table>									PRF	Number of Pulses								2	3	6	74	1000		0.1 kHz		150	270				TIE	1.0 kHz		160	153	330	1200	10100	(microjoules)	3.0 kHz		121	131	182			)
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nm-Nanometer $\lambda$ - wavelength TIE - total intraocular nsec-nanosecond																																															
kHz - kilohertz $\mu$ j - microjoule PRF - pulse repetition frequency																																															

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

# ABSTRACT

PROJECT NO. 3E7672772A813 Health Effects of Military Lasers

WORK UNIT NO. 022 System Developer Assistance Studies  
in Laser Bioeffects

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 Project MILES

STUDY NO. 3 Electrophysiological evaluations of retinal functions

STUDIES NO. 1 and 3. ED<sub>50</sub> doses (total intraocular energy (TIE) for pulse train) were determined for neodymium laser pulse trains ( $\lambda = 1060$  nm, single pulse duration = 180 nsec, single pulse ED<sub>50</sub> = 137  $\mu$ J TIE)

Pulse Repetition Frequency (PRF)	Number of Pulses				
	2	3	6	4	1000
	(energy in microjoules)				
0.1 kHz	150	270			
1.0 kHz	160	153	330	1200	10100
3.0 kHz	121	131	182		

ED<sub>50</sub> doses (TIE) were determined for Q-switch erbium laser exposures ( $\lambda = 850$  nm, pulse duration = 180 nsec) for minimum retinal irradiance diameter (ED<sub>50</sub> = 136  $\mu$ J). Five, 10, 30, and 60 second exposures were made with the prototype MILES laser (GaAs,  $\lambda = 905$  nm, single pulse duration = 110 nsec, PRF = 1.6 kHz, average power = 260 mW). Subtle retinal changes were noted with most exposures. Thirty and 60 second exposures were made with a continuous laser diode (850 nm). Retinal burns were obtained in 30 sec with 270 mJ TIE.

A feasibility pilot study was conducted in support of Project MILES (Multiple Integrated Laser Engagement System) in which evoked occipital potentials (EOP) were recorded bilaterally before and after exposures to a gallium arsenide (GaAs) laser diode and continuous wave argon laser. Changes in the EOP waveform were observed 15 to 20 min after 2 h under identical experimental procedures.

## BODY OF REPORT

WORK UNIT NO. 022

System Developer Assistance

STUDY NO. 01

Project MILES

### PROBLEM

The widespread deployment of "eye-safe" laser training devices has been in progress for the past two years. The laser of choice for this application has been the gallium arsenide diode laser. The proposed laser training device MILES (Multiple Integrated Laser Engagement Simulator) incorporates a gallium arsenide laser operating in a variety of coded pulse repetition rates. The intended field use of the MILES system requires the direct intrabeam irradiation of personnel. TRADOC proposes to field 80,000 of these devices for training of friendly troops within one-and-a-half years.

The prototype MILES laser was reviewed by the US Army Environmental Hygiene Agency in January 1976. The output was found to exceed a safe level to the eye of  $4.8 \times 10^{-8}$  J per pulse obtained by multiplying the single pulse safe level by the pulse repetition frequency (PRF) correction factor listed in TB Med 279 for 100 Hz. The PRF correction for 100 Hz was somewhat arbitrarily chosen because data do not exist for the non-uniform interpulse spacing of the gallium arsenide laser operating in the code. The device could not be classified as safe for intrabeam viewing. The dilemma presented by the USAEHA report was that while safety standards pronounce the MILES laser emission levels hazardous, available research results did not support this finding. Further research was required to clarify the effect of PRF on the ocular damage threshold, and to determine the ocular hazard of the GaAs laser wavelength (850 nm to 905 nm).

### RESULTS AND DISCUSSION OF RESULTS

The retinas of rhesus monkeys were subjected to irradiation by a prototype gallium arsenide (GaAs) laser training device. The laser wavelength was 905 nm. The laser device operated at 1600 Hz (pulse repetition frequency mode) or 132 Hz (pulse code mode) with nominal peak pulse power on one watt and 10 watts.

Rhesus monkeys weighing 2 to 3 kg were used. The animals were anesthetized and the ocular pupils dilated. The laser beam delivery system allowed continuous fundus camera observation of the retinal irradiation site during exposure. Dosimetry was accomplished prior to animal



exposure by measuring the power passing through a 7 mm aperture at the animal's eye position.

In each eye, a total of forty-eight exposures were made in a grid pattern for exposure durations from 1 sec to 90 sec.

Evaluation of the retinal sites were made by funduscopic observation, intravenous fluorescein angiography, retinal flat preparation, and/or upon imbedded serial sections for light microscopy (Trypan blue, azure II staining). These analyses were carried out for immediate, 1 h and 24 h intervals after laser exposure.

Table I lists the average power ( $P_{av}$ ), peak power ( $P_{peak}$ ), and pulse energy ( $Q$ ) incident upon the cornea for the 1 watt PRF mode, the 10 watt PRF mode, and the 10 watt pulse code mode.

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TABLE I

Laser Exposure Data

MILES GaAs Laser - 905 nm

Laser	1 watt	10 watt	10 watt
Mode	PRF	PRF	Pulse Code
$P_{av}$	34 $\mu W$	262 $\mu W$	22 $\mu W$
$P_{peak}$	0.18 watt	1.5 watt	1.5 watt
$Q$	0.212 erg/pulse	1.64 erg/pulse	1.64 erg/pulse

---

These ocular exposures did not produce the type of retinal opacity which is typically seen by funduscopy after laser irradiation of the retina. Exposures in the 30-sec PRF sequences were characterized by the development of a pale gray clouding within 10 sec after initiation of the exposure while the laser continued to irradiate the retinal site. At the end of the 30-sec interval, the exposure site measured approximately 350 to 400 microns and was darkened at the periphery with central diffuse clouding.

The incidence of observed retinal changes at various exposure levels is summarized in Table II.

---

TABLE II

## Summary of Laser Exposure Data

<u>Lasers</u>	<u>Mode</u>	MILES GaAs Laser - 905 nm		
		<u>Exposure Duration</u>	<u>Number Exposures</u>	<u>Number Changes</u>
1 watt	PRF	90 sec	2	2
		60 sec	7	6
		30 sec	109	67
		10 sec	25	18
		5 sec	7	5
		1 sec	10	0
1 watt	Pulse Code	30 sec	14	0
10 watt	PRF	30 sec	19	11
10 watt	Pulse code	30 sec	5	5

The subtle retinal changes are persistent (24 h after exposure). With the exception of the direct observation of retinal "clouding", none of the techniques (angiography, flat preparation, serial microscopy, and fundus photography) routinely used to determine the site or extent of the change demonstrated any retinal alteration.

The ocular damage threshold was determined for exposure of primate retinae to irradiation by a 850 nm erbium laser. The laser produced Q-switch pulses of 180 nsec duration in the TEM<sub>00</sub> mode. The beam diameter was 1.6 mm. The laser beam delivery system allowed fundus camera observation of the exposure site immediately before and after irradiation. Dosimetry was accomplished by a beam sampling photo diode calibrated by reference to a EGG 580 radiometer.

Forty-eight exposures were made in each eye. The criterion for damage was the presence of an ophthalmoscopically visible retinal opacity one hour after exposure. Dose-response data were processed by the technique of probit analysis to determine the ED<sub>50</sub> and associated 95% confidence limits.

Dose response data were obtained for two exposure conditions. Initial exposures used a laser beam divergence of 0.7 mrad producing a minimal

retinal irradiation diameter. After an  $ED_{50}$  had been obtained for this condition, a +9 diopter lens was introduced into the beam to produce a 26.8 mrad beam divergence resulting in a 400 micron retinal irradiation diameter. The results of the 850 nm erbium laser exposures are tabulated in Table III.

TABLE III

Summary of Laser Exposure Data

Q-Switch Erbium Laser - 850 nm

Retinal irradiance diameter	$ED_{50}$ (MJ)	95% confidence limit (MJ)
Minimum spot	12	9.5 - 15.1
400 micron	138	119-161

Retinal exposures in rhesus monkey were performed with a continuous wave GaAlAs laser at a wavelength of 850 nm. A 5.5 mm focal lens collimated the emission from the laser diode to produce a beam capable of delivering into the eye 10 mW continuous power. Measurements of the farfield pattern determined the beam divergence to be 0.6 mrad by 4.8 mrad. The projected retinal spot size for a 15 mm focal length eye was 9 microns by 72 microns. Retinal opacities were produced by 30-sec exposures. It thus appears that this laser can be used to measure a damage threshold ( $ED_{50}$ ) although this has not yet been accomplished.

Ocular damage thresholds were determined for exposure to pulse trains from a repetitively pulsed neodymium laser. These measurements were designed to determine the additivity of the effectiveness of pulses as a function of pulse number and pulse separation. Pulses exhibit total additivity if the  $ED_{50}$  dose for the pulse train is independent of the number of pulses in that train.

The laser used was a continuously pumped acousto-optic Q-switched Nd:YAG laser operating in the TEM<sub>00</sub> mode at 1060 nm. Within the pulse repetition frequency range used in these experiments, the pulse duration was 180 nsec FWHM. In all cases, the laser was pulsed continuously at the desired frequency and an external shutter was used to pass the desired number of pulses. The laser beam at the eye was 2 mm in diameter and collimated to produce a minimum retinal irradiation area.

Rhesus monkeys were used. Forty-eight exposures were made in the retina of each eye. The criterion for damage was the presence of an ophthalmoscopically visible lesion 1 h post-exposure. Dose-response



data were processed by probit analysis to determine the  $ED_{50}$  and associated 95% confidence limits. Table IV is a summary of the results of this study.

TABLE IV

Laser Exposure Data

Neodymium Laser Pulse Trains  
1060 nm

<u>Pulse Repetition Frequency</u>	<u>Number of Pulses</u>					
	1	2	3	6	74	1000
100 Hz $ED_{50}(\mu J)$	137.5	75.1	89.8			
1000 Hz $ED_{50}(\mu J)$		80.1	51	55	16.4	10.1
3000 Hz $ED_{50}(\mu J)$		60.6	43.6	30.4		

These data show a high degree of additivity for two pulses, with the additivity being greater for shorter interpulse spacing. The degree of additivity lessens for pulses after the second. If the data for 1000 Hz are plotted as total pulse train or energy vs total exposure duration on Log-Log paper, the slope of the resulting line is nearly identical to the slope of existing continuous wave (cw) neodymium  $ED_{50}$  data. This is at variance with the guidance of TB Med 279 which assumes a different time dependency of the safe level for the two exposure conditions. This variance encouraged the investigators to perform a literature search for all existing pulse train exposure data. Examination of these data, for pulse durations from 10 nsec to 1 msec and pulse repetition frequencies from 1 Hz to 10000 Hz reveal a consistent relationship between pulse train data and equivalent continuous wave data which is dependent upon pulse duration and pulse repetition frequency. This relationship is not adequately modeled by the procedures of TB Med 279. The investigators (D. Lund and B. Stuck) are preparing a document which delineates the relationship between pulse train  $ED_{50}$  data and cw laser  $ED_{50}$  data and recommends a modified procedure for computation of the maximum permissible exposure level for laser pulse trains.

CONCLUSIONS

The prototype MILES GaAs laser device is capable of producing retinal alterations. These alterations are not like the lesions typically

seen as a result of laser exposure. Their nature and significance is not yet understood.

The procedures of TB Med 279 for calculation of the maximum permissible exposure to laser pulse trains does not accurately model existing damage threshold data. Recommendations for modification of these procedures will be formally submitted.

#### RECOMMENDATIONS

Further studies are needed with the pulsed GaAs diode to determine the nature and significance of the retinal alteration observed. The ocular damage threshold should be determined for the cw GaAs laser. More data are required on the ocular hazard of pulse trains at a variety of pulse durations, pulse repetition rates and wavelength. Especially essential are data for pulse durations of 1 to 10  $\mu$ sec. Pulse durations in this time domain appear to be more hazardous than either longer or shorter pulse durations.

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## BODY OF REPORT

WORK UNIT NO. 022

System Developer Assistance

Studies in Laser Bioeffects

STUDY NO. 03

Electrophysiological evaluations  
of retinal functions

### PROBLEM

The retinal appearance following laser irradiation has been well documented. What has remained difficult to ascertain, however, is the effect upon vision following low level (below the threshold for an ophthalmoscopically visible lesion) laser exposures at wavelengths currently being used in the field. Gallium arsenide (GaAs) lasers which operate at 850 nm have previously been considered "eye-safe" systems. However, recent findings of this laboratory have indicated a subtle change in the appearance of the retina following exposure to this laser.

The purpose of the feasibility pilot study was to evaluate further the observed changes in the retina with changes in the electrophysiological characteristics of the visual system following exposure to a prototype GaAs diode which may be incorporated into the Project MILES system.

### RESULTS AND DISCUSSION OF RESULTS

The integrity of the visual system can be inferred in four ways. Ophthalmoscopic techniques in which changes are seen but their significance is not directly apparent. Behavioral techniques in which the animal is required to perform certain vision-related tasks but generally will not allow a quick response to the question of whether the animal can "see." The training is long and costly. Electroretinography (ERG) in which the integrity of the retina as a whole is evaluated is a good quick technique. However, the plethora of retinal elements unaffected by the laser exposure tends to mask the effects of a small sized retinal change. The evoked occipital potential (EOP), which has not been widely used to determine retinal dysfunction, may yield clues as to changes in the visual system following retinal exposures.

In the present study, one animal pre-anaesthetized with ketamine and maintained under pancuronium -Br, was presented with a large image of a grating. The grating was inserted into the optics of a Ziess fundus camera and projected onto the retina of the dilated eye. In the first run, EOPs were recorded from each occipital lobe when alternately, the left, right, left and right eyes were stimulated. An exposure to the right eye of  $7.4 \times 10^{-3}$  J of total intraocular energy (TIE) for a 16 msec



argon laser pulse (514.5 nm) was made and several thousand evoked potentials were then obtained. The averaged (N=64) potentials showed no immediate change from its pre-exposure amplitudes or latencies. Changes in the shape of the EOP were observed 15 to 20 min post exposure in the contralateral cortex. None were seen ipsilaterally, nor were any retinal changes observed during this period. The changes persisted until the end of the run (2 h).

Examination of the right eye one week later showed a small (500 $\mu$ ) irregular lesion superior and nasal to the fovea in the macula. Following the same format as above, the left eye was then exposed to GaAs radiation for 60 sec. Average intraocular power was 260 mW with pulse repetition rate of 1600 Hz. As before, waveform changes were seen after approximately 20 min post exposure, however, this time they occurred bilaterally. Examination showed no visible retinal changes.

A follow-up sham study in which the EOP was obtained bilaterally, showed no waveform changes after 2 h of grating presentation.

The meaning and implications of the observed changes in terms of the visual system are difficult to determine at this time. The appearance of waveform alterations in the contralateral cortex, together with bilateral changes during the second run, indicate that we have a possible extremely sensitive tool to investigate suspected retinal dysfunction due to laser irradiation.

#### CONCLUSIONS

An apparently very sensitive, relatively easy technique has been utilized to determine changes in a small retinal area induced by laser radiation.

#### RECOMMENDATIONS

After thresholds have been obtained for some visible, and near IR EOP changes, we will be able to evaluate quickly functional disturbances of the visual system. Correlative studies, utilizing animal behavior, ERG, and EOP should be conducted so that a more direct relationship can be established between EOP waveform changes and actual vision changes.

#### PUBLICATIONS

1. RANDOLPH, D. I., and B. E. STUCK. Sensitivity of the rhesus monkey cornea and surrendering tissues to heat produced by CO<sub>2</sub> laser radiation. In: Proceedings of the 1976 Army Science Conference (West Point, N.Y., 1976)
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 77 07 19	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>a</sup> NA	8. DISSEM INSTRN <sup>a</sup> NL	9a. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62177A		3E162172A813		00	
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11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Biological Investigations in Prediction and Protection Against Coherent Radiation							
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c. TYPE Not Applicable				FISCAL YEAR		280	
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: <sup>a</sup> Letterman Army Institute of Research				NAME: <sup>a</sup> Letterman Army Institute of Research			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
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				NAME: Zwick, H., DAC			
				NAME: Randolph, D. I., DAC POC:DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Laser Systems Safety; (U) Laser Hazard; (U) Eye Damage; (U) Vision							
23. TECHNICAL OBJECTIVE, <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) The objectives are to determine the effects on vision of repetitive low level laser radiation at and below "safe" exposure levels, and to provide these data with recommendations to USAEHA for further refinement of Army Safety Standards, the DARCOM, developer and Army user for consideration in development of laser systems. 24. (U) Behavioral and neurophysiological techniques, adapted for rhesus, are used for visual testing. Temporary and/or permanent effects correlated with morphological changes most realistically represent the actual hazards of laser radiation. These measures provide the data base for safety standards revision. 25. (U) 7610-7709. Sensory detection of far 10.6 micron laser radiation by the skin was demonstrated at irradiance near 10 mw/cm <sup>2</sup> for a beam diameter of 16 mm and 20 sec exposure. This irradiance is 10 times below the maximum permissible exposure for these exposure conditions. For repeated exposures at and below the maximum permissible exposure of 100 mw/cm <sup>2</sup> (for 1200 exposures of 20 sec duration) no direct ocular tissue alteration has been observed by slit lamp examination. In repeated exposures to argon laser radiation, visual acuity, and spectral sensitivity were permanently altered following successive intermittent 2-hour exposure periods totaling 38 hours. The average irradiance on the diffuser was 25 $\mu$ w/cm <sup>2</sup> . This irradiance is more than 100 times below "safe" levels for viewing of a diffuse extended source. Retinal function has been evaluated following laser irradiation by utilizing the evoked occipital potentials of the rhesus. Changes in both the amplitude and latencies of these waveforms have been observed after a single argon laser exposure of 7.4 millijoules for a spot size of 500 $\mu$ (16 milliseconds)							

<sup>a</sup> Available to contractors upon originator's approval.



# ABSTRACT

PROJECT NO. 3E7672772A813 Health Effects of Military Lasers  
WORK UNIT NO. 025 Biological Investigations in Prediction and Protection Against Coherent Radiation

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 Effects of laser irradiation on visual function

STUDY NO. 2 Detection of heat at the cornea by rhesus monkeys

In repeated exposures to argon laser radiation, visual acuity, and spectral sensitivity were permanently altered following successive intermittent 2 h exposure periods totaling 38 h. The average irradiance on the diffuser was  $25 \mu\text{W}/\text{cm}^2$ . This irradiance is more than 300 times below "safe" levels for viewing of a visible extended source. Retinal function has been evaluated following laser irradiation by utilizing the evoked occipital potentials of the rhesus. Changes in both the amplitude and latencies of these waveforms have been observed after a single argon laser exposure of 7.4 mJ for a spot size of  $500 \mu$  (16 msec). Present ongoing research has shown that laser light is different from other types of light when equated for wavelength and irradiance. The effects appear to be due to coherency and bandwidth.

Sensory detection of far infrared 10.6 micron laser radiation by the skin was demonstrated at irradiance near  $10 \text{ mW}/\text{cm}^2$  for a beam diameter of 16 mm and 20 sec exposure. This irradiance is 10 times below the maximum permissible exposure for these exposure conditions. For repeated exposures at and below the maximum permissible exposure of  $100 \text{ mW}/\text{cm}^2$  (for 1200 exposures of 20 sec duration) no direct ocular tissue alteration has been observed by slit lamp examination.

## BODY OF REPORT

WORK UNIT NO. 025

Biological Investigations in Prediction and Protection Against Coherent Radiation

STUDY NO. 1

Effects of laser irradiation on visual function

### PROBLEM

Present laser safety standards are based primarily on gross morphological criteria. Changes in vision, the process of "seeing" associated with laser radiation have not been incorporated into these standards. The purpose of this research is to provide data on such changes, correlation of such changes with morphological criteria, and thereby to provide future laser safety standards based on functional as well as morphological criteria.

### RESULTS AND DISCUSSION

Low level exposure to 514 nm radiation has produced a permanent change in the spectral sensitivity of the rhesus. The level used in this experiment was 300 times below the current safe level for visible extended source criteria. Furthermore, this effect was not immediate but dependent upon repetition, so that only after 18 h of cumulative exposure did the first evidence of alteration occur. Post-exposure measurements, continuing for seven months, revealed permanent suppression of photopic spectral sensitivity. Little change in scotopic sensitivity occurred, which indicates that the effect as measured is a dominantly cone function. Evidence of scotopic enhancement however, was obtained during the latter hours of exposure, so that the suppression of cone activity observed may affect scotopic vision by altering normal photopic-scotopic neural interaction processes.

Because the levels of 514 nm light used in this experiment were much lower than other experiments performed, an experiment was set up to compare directly the effects of laser and non-laser light of equal energy and wavelength. These experiments are presently in progress but early data have definitely demonstrated that laser light is far more effective in producing changes in electroretinographic(ERG) spectral sensitivity that are permanent and reflect photoreceptor system alteration. Preliminary investigations suggest that both the bandwidth and possibly the coherency of laser light are responsible for these low level laser effects on visual function.

Low level effects of near infra-red gallium arsenide prototype laser training device was evaluated for its effect on ERG spectral sensitivity. Changes in spectral sensitivity of a relatively permanent nature

were obtained. These effects are currently being followed up by using evoked cortical potential techniques. Correlation of these changes with low level gallium arsenide retinal "clouding" changes is being pursued.

The problem of multiline visible laser devices and presently available methodology for protection from such devices was considered regarding the copper vapor laser. To protect the eye adequately from such a device, a considerable temporary reduction in visual sensitivity is necessary. The effects of such devices on visual function required for many military tasks are serious and serious consideration of this protection problem is warranted.

Development of a task to measure both static and dynamic acuity in the rhesus monkey was completed. This task makes it possible to measure the spectral sensitivity for acuity criteria for both the peripheral and central retina with the use of Landolt ring acuity criteria. Two animals are presently trained and will shortly be exposed to 514 nm low level laser radiation.

#### CONCLUSIONS.

Data from functional criteria indicate that permanent changes in these measures can occur at levels many times below those of gross morphological criteria. Such changes seem to be correlated with ultrastructural changes in photoreceptors. Laser light effects on visual function are far more potent than that of ordinary incoherent light. Protection problems will become extremely serious as multiline visible laser sources proliferate.

#### RECOMMENDATIONS

1. Data base for low level light effects should be extended. Four new animals are being trained, two of which can be studied for dynamic as well as static acuity functions. Direct electronmicroscopic/ psychophysical correlation study should be conducted.
2. Continuation and expansion of studies should be designed to differentiate effects from coherent light and non-laser light. These studies are essential as more and more interest in comparison of laser and non-laser light standards grows.
3. Protection problem should be attacked vigorously, especially with respect to multiline lasers. New materials and/or biological methods of protection need to be explored.



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## BODY OF REPORT

WORK UNIT NO.	025	Biological Investigations in Prediction and Protection Against Coherent Radiation
STUDY NO.	02	Detection of heat at the cornea by rhesus monkeys

### PROBLEM

The carbon dioxide ( $\text{CO}_2$ ) laser radiation which operates in the far infrared (IR) portion of the electromagnetic spectrum is increasingly being employed in field tactical situations. The possibility exists that while a single, short exposure to 10.6 microns at or below the "safe" level may not result in tissue changes, exposure for many doses over a long period of time, may be cumulative.

The purpose of this research was to determine the long-term effects of  $\text{CO}_2$  laser radiation upon the behavior of trained rhesus monkeys.

### RESULTS AND DISCUSSION OF RESULTS

In this study, two animals were trained by a modification of the conditioned response suppression technique. The major findings of this study are stated in following paragraphs.

The cornea as an organ for detecting the presence of  $\text{CO}_2$  radiation at 10.6 microns is relatively insensitive when compared to the lids and surrounding ocular tissues. At the highest energy levels used in the present study ( $200 \text{ mW/cm}^2$ ) which was twice the "safe" level as promulgated by TB Med 279, doubtful positive responses were obtained when a 4 mm diameter beam was directed to the cornea. In those instances where positive responses were recorded to corneal stimulation, they occurred 12 to 15 sec after the onset of the stimulus. Two confounding factors were hypothesized. First, the cornea of the rhesus (as is the human) is covered by a tear layer. Exposure of the cornea to temperature elevations of approximately  $5^\circ\text{C}$  produced by an irradiance of  $200 \text{ mW/cm}^2$  results in accelerated evaporation of the tear film, and consequently, a slight drying of the corneal epithelium. This drying action, when observed in humans, results in local irritation due to stimulation of free nerve endings in the corneal epithelium, which then triggers the blink reflex. At  $200 \text{ mW/cm}^2$ , the irradiance is quickly detected at the lids. To test this hypothesis, the eye of an anesthetized rhesus monkey was observed under slit-lamp illumination. When the cornea was exposed to room temperature, little or no breakup of the tear film was observed until 35 to 40 seconds had elapsed. However, when the animal was subjected to an irradiance of

200 mW/cm<sup>2</sup> for a 20 sec period, the tear film was seen to breakup within 10 to 15 sec after the onset of the exposure. A second explanation of the observed corneal responses to radiation is that the general gross eye movements of the awake, head-restrained animal could result in exposure of a different area of the anterior surface of the eye (i.e., the scleral or conjunctival tissue) which may be more sensitive to increases in temperature than the cornea.

Rhesus monkeys responded to CO<sub>2</sub> irradiance levels at the surface of skin ten times lower than the previously established "safe" levels as promulgated by TB Med 279 and other laser safety standard publications. This finding, together with a substantial agreement of rhesus and human exposure data, indicated that the presence of low intensity radiation at 10.6 unit could be sensed. The importance of this finding lies in the potential use of CO<sub>2</sub> lasers as tracking, designating or homing devices during the target acquisition, identification and firing phases of a battlefield engagement. Since it is to be expected that the beam diameter of CO<sub>2</sub> lasers used in the above context will exceed one square meter, it is conceivable that the sensation of a sudden "warming" of tissues could result in the abortion or delay of a mission-related task.

An important finding from a "safety" aspect of this previous study was that after many hundreds of 20-second exposures at 200 mW/cm<sup>2</sup> irradiances, no changes have been observed in the corneas of either animal when slit-lamp examinations were performed. This irradiance level represents twice the "safe" viewing level. To support this observation further, two untrained rhesus monkeys were exposed to one 20-second and three 20-second exposures respectively. The animals were then immediately sacrificed and excised corneas stained with Trypan blue and examined microscopically. The tissues appeared normal in each case.

An animal behavioral training technique was developed in which the rhesus not only was able to signify the presence or absence of CO<sub>2</sub> laser radiation, but reasonable accurate relationships between response time, irradiance, and a real stimulation were obtained. The interactions of these variables on the skin agreed closely with those found in humans when near infrared "heat lamps" were used as stimuli.

Data from the earlier study have proven extremely useful thus far in evaluating the behavior of CO<sub>2</sub> laser irradiated rhesus monkeys. The emphasis of the research up to this time has been directed both toward describing the minimum detectable irradiance variables and upon perfecting behavioral techniques.

#### CONCLUSIONS

We have demonstrated that rhesus monkeys can be trained to detect CO<sub>2</sub>



laser radiation and, at the levels used, no significant tissue effects were observed.

#### RECOMMENDATIONS

Recent information concerning the impending uses of CO<sub>2</sub> radiation now requires an evaluation of both the long and short-term effects of laser exposures at increments above the safe levels. To this end, data are needed on the functional effects of exposures above the ED<sub>50</sub> irradiance levels. Corneal epithelial tissue is particularly vulnerable to CO<sub>2</sub> radiation. At these higher levels, it is hypothesized that epithelial tissues will become edematous following laser exposure. As the cornea swells, visual acuity will suffer. By re-training the two rhesus monkeys used in the earlier study to detect the gap in a medium contrast landolt acuity "C", variations in visual acuity can be measured as a function of corneal involvement at each irradiance level. Concomitant slit lamp and specular microscopy, together with keratometry and pachometry should be correlated with dose level and visual acuity changes. With this knowledge, protective and/or treatment techniques may be developed.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>a</sup> NA	8A. DR&E INSTN <sup>a</sup> NL	8B. SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT	
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3S162172A814		00	001			
b. <del>SECONDARY</del>	62772A	3S762772A814		00	001			
c. <del>THIRDARY</del>	CARDS 114 f							
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) In Vitro Metabolism of RBCs Stored in CPD with 193.1mM Glucose and 2.05mM Adenine								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 002300 Biochemistry; 003500 Clinical Medicine								
13. START DATE 74 11		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (In thousands)
a. DATES/EFFECTIVE:		EXPIRATION:		PRECEDING				
b. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		2.0		60
c. TYPE:		d. AMOUNT:		CURRENCY				
e. KIND OF AWARD:		f. CUM. AMT.		78		2.0		52
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research				
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Blood Research Division				
				ADDRESS <sup>a</sup> Department of Surgery				
				ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> Bensinger, Thomas A., LTC, MC				
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875				
				SOCIAL SECURITY ACCOUNT NUMBER:				
21. GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign Intelligence Not Applicable				NAME: Peck, Carl C., LTC, MC				
				NAME: Moore, Gerald L., PhD, DAC POC: DA				
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Erythrocyte Life Prolongation; (U) CPD Adenine; (U) Blood Storage; (U) ATP; (U) Glucose								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Glucose levels of 128.7 mM in citrate phosphate dextrose (CPD) adenine will not maintain adenosine triphosphate (ATP) at levels greater than 2.0 µM/gHb in 90% packed erythrocytes (RBC) for 35 days of storage at 4 C. By increasing the glucose to 257.4 mM in CPD adenine, ATP was maintained at greater than 2.0 µM/gHb for 5 weeks. It is important to determine if a smaller concentration of glucose, i.e., 193.1 mM (1.5 times normal) or less in CPD, supplemented with 2.05 mM adenine will have sufficient glucose for maintenance of ATP at greater than 2.0 µM/gHb after 35 days of storage. This work is important to the Army's continuing effort to extend the shelf-life of stored blood.  24. (U) The levels of glucose, ATP, 2,3-diphosphoglycerate, and pH will be determined in blood stored in the CPD.  25. (U) 76 10 - 77 09 A 42-day blood storage experiment employing two hematocrit levels (40%, 80%) three anticoagulant adenine levels (1.48, 2.05, 3.03 mM), and three anti-coagulant glucose levels (160.9, 193.1, 225.2 mM) has been completed. Analysis of these data suggests further experimentation is necessary to pin-point the optimal anti-coagulant glucose/adenine levels for 35-day storage of whole blood (Hct=40%) and red cell concentrates (Hct=80%).								

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 001 In Vitro Metabolism of RBCs Stored  
in CPD with 193.1mM Glucose and 2.05mM  
Adenine

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 In vitro metabolism of packed erythrocytes stored  
at various glucose concentrations in CPD-adenine

To determine more precisely the optimum amount of adenine and glucose necessary to allow maintenance of adenosine triphosphate (ATP) at greater than 2  $\mu\text{m/gm}$  Hb following 35 days of 4 C storage, studies have been completed in which the blood storage bags containing citrate-phosphate-dextrose (CPD) supplemented with 160.9, 193.1, or 225.2 mM glucose and 3 levels of adenine: 1.49 mM, 2.05 mM, or 3.03 mM. A minimum of 225.2 mM glucose and 3.03 mM must be present in CPD to insure that greater than 95% of packed cell units (Hct 80) will exhibit ATP levels greater than 2  $\mu\text{m/gm}$  Hb following 35 days of storage.



## BODY OF REPORT

WORK UNIT NO. 001

In Vitro Metabolism of RBCs Stored in CPD with 193.1mM Glucose and 2.05mM Adenine

STUDY NO. 1

In vitro metabolism of packed erythrocytes stored at various glucose concentrations in CPD-adenine

### PROBLEM

Prolongation of blood storage capabilities beyond the current limit of 21 days would significantly enhance medical care of wounded soldiers. Citrate-phosphate-dextrose (CPD) has been established as a useful anti-coagulant for liquid blood storage up to 21 days and, with adenine supplementation, CPD will permit storage of red blood cells for 35 days. European formulas for CPD-adenine have contained 4.10 mM adenine. Since adenine may cause renal toxicity, it is desirable to develop a formula for CPD which would contain the least amount of adenine that will allow storage in the liquid state for 35 days. Studies of packed cells stored in CPD-adenine have indicated that glucose in excess of that in standard CPD (128.7 mM) may be necessary for adequate maintenance of erythrocyte ATP ( $>2 \mu\text{m/gm Hb}$ ) through 35 days' storage. Thus, a systematic search for the optimal glucose and adenine content of CPD preservative is necessary.

### RESULTS AND DISCUSSION OF RESULTS

A systematic study of packed cells (Hct 80) stored for 35 days (4 C) in CPD preservative with 3 glucose levels (160.9, 193.1, 225.2 mM), each at 3 adenine levels (1.47, 2.05, 3.03 mM), has been completed. Erythrocyte ATP levels are shown in Table I. The right-hand column in Table I represents estimates of the percentage of packed cell units which would exhibit red cell ATP of greater than  $2 \mu\text{m/gm Hb}$  following 35 days storage. It can be seen that only in CPD containing 225.2 mM glucose and 3.03 mM adenine would one expect greater than 95% of such packed cell units to have red cell ATP levels in excess of  $2 \mu\text{m/g Hb}$ .

The results of this study were presented and discussed at a recent meeting at the Bureau of Biologics (FDA) of military, civilian, and industry researchers interested in developing improved blood preservatives (see Annual Report, FY 77, Work Unit No. 004). It was the consensus of that group that 42-day storage studies of improved CPD adenine preservatives should be initiated with the use of CPD containing 225.2-257.4 mM glucose and 4.10 mM adenine.

TABLE I

Red Cell ATP in Packed Cells Following 35 Days Storage

Anticoagulant Content Glucose (mM)	Adenine (mM)	n	ATP ( $\pm$ SD) ( $\mu$ m/gm Hb)	Estimated <sup>†</sup> % age of PC <sup>††</sup> Units With ATP >2 $\mu$ m/gm Hb
160.9	1.47	3	2.29 $\pm$ 0.47	70
	2.05	3	2.08 $\pm$ 0.22	60
	3.03	3	2.64 $\pm$ 0.75	75
193.1	1.47	2	1.72 $\pm$ 0.06	10
	2.05	3	2.41 $\pm$ 0.24	88
	3.03	3	1.48 $\pm$ 0.56	75
225.2	1.47	3	2.23 $\pm$ 0.19	85
	2.05	3	2.93 $\pm$ 0.44	90
	3.03	3	3.07 $\pm$ 0.34	95

<sup>†</sup>Estimation based on percentiles of the Student's t-distribution for n,  $\bar{X}_{ATP}$  and  $SD_{ATP}$  in adjacent column

<sup>††</sup>PC = packed red cells (Hct 80)

#### CONCLUSIONS

A minimum of 225.2 mM glucose and 3.03 mM adenine must be present in CPD anticoagulant-preservative to ensure that greater than 95% of packed cell units (Hct 80) exhibit erythrocyte ATP levels of greater than 2  $\mu$ m/gm Hb.

#### RECOMMENDATIONS

In vitro experimentation with CPD-adenine anticoagulant-preservatives should be performed by using glucose equal to or in excess of 225.2 mM and adenine up to 4.10 mM. Effects of these modifications of CPD should be studied on whole blood and packed red cells stored for 42 days and on platelets stored for 72 hours at 22 C.

#### PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&F(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DESGN INSTR <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT
77 07 19	H.Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
6. PRIMARY	62172A	3S162172A814	00	002			
7. SUBPRIMARIES	62772A	3S762772A814	00	002			
8. CONTINUANCES	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) In Vitro Metabolism of G6PD Deficient Erythrocytes Stored in CPD Adenine Anticoagulant							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008800 Life Support; 003500 Clinical Medicine; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 03		77 09		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		34	
C. TYPE:				CURRENT			
D. KIND OF AWARD:				78		0.0	
E. CUM. AMT.						00	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Department of Surgery			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> Bensinger, Thomas A., LTC, MC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) G6PD Deficiency; (U) Ascorbate;							
(U) Blood Storage; (U) Adenine; (U) CPD; (U) G6PD							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) Ten percent of the American Negro male population is heterozygous for glucose-6-phosphate dehydrogenase (G6PD) deficiency, and this population makes a significant contribution to the blood donation program of the U.S. Army. Hence, military blood banks may contain a significant amount of G6PD deficient blood. This study will determine the ability of G6PD deficient erythrocytes to maintain metabolic integrity during 35 days of storage in CPD-A<sub>1</sub> (citrate-phosphate-dextrose with 2.05 mM adenine and 160.9 mM glucose).</p> <p>24. (U) Blood will be drawn from identified G6PD deficient donors and stored in the CPD-A<sub>1</sub> anticoagulant media at 4 C. At appropriate time intervals, samples will be removed aseptically from these bags, and tests for adenosine triphosphate (ATP), reduced glutathione (GSH), and Heinz body formation will be undertaken.</p> <p>25. (U) 76 10 - 77 09 A total of seven units of G6PD deficient blood have been stored in a variety of anticoagulant media including CPD, CPD-A<sub>1</sub>, CPD-ascorbate, and CPD-A<sub>1</sub>-ascorbate. G6PD deficient cells do not respond as well to adenine in maintaining ATP as do nondeficient erythrocytes; however, they do possess higher ATP levels in comparison to G6PD deficient cells not stored in adenine media. Ascorbate does appear to promote 2,3 diphosphoglycerate (2,3-DPG) maintenance in G6PD deficient cells as it does in non-deficient cells. An additional finding is an excess accumulation of pyruvate in G6PD deficient blood when stored in all four anticoagulants when compared to normal controls in the same storage media. This increased pyruvate may result from reduced nicotinamide adenine dinucleotide phosphate (NADPH) levels in G6PD deficient cells. NADPH is a necessary cofactor for lactate dehydrogenase (LDH) when the pH decreases during storage. This work unit is being terminated due to departure of the principal investigator and lack of need for further studies at this time.</p>							

DD FORM 1498

1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 002 In Vitro Metabolism of G6PD Deficient  
Erythrocytes Stored in CPD Adenine  
Anticoagulant

The following investigations have been conducted under this Work Unit:

STUDY NO. 2 Measurement of some intermediate metabolites and enzyme activity of G6PD deficient erythrocytes stored in CPD, CPD-adenine, CPD ascorbate, and CPD adenine ascorbate anticoagulant media when compared to normal, nondeficient erythrocytes stored in the same media

Experimental work completed in this study was summarized in the Annual Progress Report for FY 76. The only work done on Work Unit 002 during this fiscal year was preparation of a manuscript. This work unit is being closed out.



## BODY OF REPORT

WORK UNIT NO. 002

In Vitro Metabolism of G6PD Deficient Erythrocytes Stored in CPD Adenine Anticoagulant

STUDY NO. 2

Measurement of some intermediate metabolites and enzyme activity of G6PD deficient erythrocytes stored in CPD, CPD adenine, CPD ascorbate, and CPD adenine ascorbate anticoagulant media when compared to normal, nondeficient erythrocytes stored in the same media

### PROBLEM

Since 10% of the American Negro male population is deficient for glucose-6-phosphate dehydrogenase (G6PD), it is important to determine if erythrocytes from these donors behave in the same manner metabolically as erythrocytes from normal controls when stored under identical conditions. Since approximately 25-30% of all military personnel are black, the possible adverse effects of G6PD deficient blood donors could be especially severe in the U.S. Army. The newer additives, such as adenine or ascorbic acid, which prolong liquid storage to 35-42 days could cause additional stress on G6PD deficient blood. Therefore, this study was undertaken to evaluate what, if any, changes occur during 6 weeks of storage.

### RESULTS AND DISCUSSION OF RESULTS

No additional experimental work was done on this work unit in the past year. A manuscript was prepared based upon data collected the previous year. All experimental work completed in this study was summarized in the Annual Progress Report for FY 76. This work unit is being terminated.

### CONCLUSIONS

The only biochemical difference between normal and G6PD deficient cells during storage is elevated levels of pyruvate in the G6PD deficient cells. Although in vivo survival studies were not done, it appears from the biochemical data (including ATP, 2,3-DPG, and pH) that G6PD deficient cells survive 4 C liquid storage as well as normal red blood cells, and G6PD deficient donors should be accepted as part of the normal donor pool.

### RECOMMENDATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6091	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DISSEM INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62172A	3S162172A814		00	003		
b. CONTRIBUTORS	62772A	3S762772A814		00	003		
c. CONTRIBUTORS	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Preservation of 2,3-DPG in Red Cells Subjected to Extended Liquid Storage							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008800 Life Support; 003500 Clinical Medicine; 002300 Biochemistry; 002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 03		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		77	
c. TYPE:				CURRENT		2.0	
d. KIND OF AWARD:				78		0.2	
e. CUM. AMT.						94	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Blood Research Division			
				Department of Surgery			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> Moore, Gerald L., PhD, DAC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Oxygen Transport; (U) Massive Transfusions;							
(U) Blood Storage; (U) 2,3-DPG; (U) Hemoglobin Function; (U) Ascorbate-2-PO <sub>4</sub>							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)							
23. (U) Although the significance at the tissue level of a left-shifted oxygen dissociation curve exhibited by 2,3 diphosphoglycerate (DPG) deficient red cells is not completely clear, it is highly probable that red cells with near normal DPG content would contribute to organ survival in the massively transfused, acutely wounded soldier. Currently used red cell storage anticoagulant-preservative solutions (APS) do not permit the red cell to maintain DPG content. Normal oxygen off-loading characteristics of hemoglobin are dependent, at least in part, on the allosteric regulation of DPG within the red cell. These studies will develop and evaluate both additive and storage systems which will enable red cells to maintain DPG better during liquid storage, without impairing other glycolytic intermediates such as adenosine triphosphate (ATP).							
24. (U) Most APS additives which currently appear to hold promise for improving red cell DPG during storage are unstable in the anticoagulant mix. Among these are dihydroxyacetone (DHA), ascorbic acid (AA), and bicarbonate (BC). Stable forms or systems employing these substances will be developed and tested. The stable forms must be inert to the plastic blood container, freely convertible to the active form upon contact with whole blood, and nontoxic both to the blood in storage and the recipient on infusion.							
25. (U) 76 10 - 77 09 Liquid storage of "anticoagulant-DHA" is unstable in citrate-phosphate-dextrose (CPD) or phosphate, but is stable in water or saline at room temperature. All systems are unstable at 50 C. The chemistry of DHA breakdown has been elucidated. Various concentrations of ascorbate-2-PO <sub>4</sub> (0 to 42 mM) in blood were evaluated for their efficacy in maintaining 2,3-DPG; 30 mM was found most satisfactory.							

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 003 Preservation of 2,3-DPG in Red Cells  
Subjected to Extended Liquid Storage

The following investigations have been conducted under this Work Unit:

STUDY NO. 2 Stabilization of ascorbic acid as a red cell preservative

STUDY NO. 5 The stabilization of dihydroxyacetone (DHA) for use in maintaining red cell 2,3-DPG during liquid storage

Currently available techniques for the liquid preservation of red cells do not maintain the red cell 2,3 diphosphoglycerate (2,3-DPG) concentration sufficiently so that it can modulate hemoglobin to release oxygen optimally. This defect of stored red cells may be of great importance in hemorrhagic shock, and thus, in resuscitation of the massively wounded soldier.

STUDY NO. 2. Units of blood drawn in CPD-adenine were fortified with 0-41 mM ascorbate phosphate (AsP). The 2,3-DPG concentrations were best maintained with 41 mM AsP (73% of normal on storage day 35, and 41% on day 42). Addition of dihydroxyacetone to 41 mM AsP did not further enhance 2,3-DPG maintenance.

STUDY NO. 5. The stability of DHA in various aqueous solutions was evaluated for 13 months at 25 C. DHA is stable in water and saline, as well as saline solutions of adenine or dextrose. It is unstable in other anticeagulant compounds or in any solution at 50 C (2 months). The effect of autoclaving was evaluated. Optimal concentration of DHA when added to CPD-adenine whole blood is being evaluated. Several derivatives of DHA have been prepared and are being tested in blood.



### BODY OF REPORT

WORK UNIT NO. 003

## Preservation of 2,3-DPG in Red Cells Subjected to Extended Liquid Storage

STUDY NO. 2

## Stabilization of ascorbic acid as a red cell preservative

## PROBLEM

After 14 days of liquid storage as whole blood or red cell concentrates, red cell 2,3-DPG is not maintained at levels permitting normal oxygen transfer to tissues. Preliminary data suggest that decreased oxygen transfer may be particularly deleterious to soldiers requiring massive transfusions to correct hemorrhagic shock. It has recently been found that ascorbic acid, especially in combination with dihydroxyacetone (DHA) will maintain 2,3-DPG levels above 50% during extended liquid storage (35 days). However, ascorbate, either as the acid or as the sodium salt, is not stable in solution and decomposes rapidly when exposed to the autoclaving necessary to sterilize blood bags.

It is important, therefore, to develop ways of stabilizing this compound. The stability and efficacy of derivatives of ascorbic acid such as ascorbate-2-phosphate (AsP) will be evaluated.

## RESULTS AND DISCUSSION OF RESULTS

Ascorbate-2-phosphate (AsP) has been shown to be 80% stable to autoclaving and to survive 6 months of 25 C liquid storage in water or saline. Addition of AsP to CPD-adenine whole blood resulted in increased maintenance of red cell 2,3-DPG throughout 42 days of storage. AsP concentrations of 0 to 41 mM were used; the highest concentration gave the best 2,3-DPG preservation. About 50% of the AsP disappeared from the plasma during the 42-day storage period. Addition of DHA to bags containing 41 mM AsP did not enhance 2,3-DPG maintenance as was previously reported for ascorbic acid plus DHA; this needs further evaluation.

## CONCLUSIONS

AsP is stable to autoclaving and can be stored for 6 months at 25 C in aqueous solution. While AsP enhanced 2,3-DPG maintenance during storage, it did not show an additive effect when used in combination with DHA.



### RECOMMENDATIONS

Further studies should be performed on the storage stability of AsP and its effects on maintaining red cell 2,3-DPG during storage.

### PUBLICATIONS

1. BENSINGER, T.A., and T.F. ZUCK. Trisodium ascorbate phosphate, a stabilized form of ascorbic acid, promotes 2,3-DPG maintenance during blood storage. (Abstract) Transfusion 16:518, 1977
2. BENSINGER, T.A., T.F. ZUCK, B. TOLBERT, S. MCLAUGHLIN, C.C. PECK, and M. KNIGHT. An enzymatic method for measurement of ascorbate-2-phosphate. Biochem Med, in press, 1978

STUDY NO.	5	The stabilization of dihydroxyacetone (DHA) for use in maintaining red cell 2,3-DPG during liquid storage
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### PROBLEM

After 14 days of liquid storage as whole blood or red cell concentrates, red cell 2,3-DPG is not maintained at levels permitting normal oxygen transfer to tissues. Preliminary data suggest that decreased oxygen transfer may be particularly deleterious to soldiers requiring massive transfusions to correct hemorrhagic shock. It has recently been found that ascorbic acid, especially in combination with dihydroxyacetone (DHA) will maintain 2,3-DPG levels above 50% during extended liquid storage (35 days). The additive DHA appears to be unstable in solution with anticoagulants or when autoclaved.

It is important, therefore, to develop ways of either stabilizing this compound, or to develop modified compounds which have the same potential effect as DHA in preserving red cell 2,3-DPG. It is necessary to study the rate and mechanism of DHA decomposition in various aqueous solutions and to develop a method for its stabilization. Stabilization of DHA may be achieved by development of a complex or derivative.

### RESULTS AND DISCUSSION OF RESULTS

The 150 mM solutions of DHA in sterile blood transfer packs were stored at 25 C for 13 months. DHA was stable in water, saline, saline plus adenine or dextrose, or 16 mM phosphate. DHA was unstable in CPD, citrate, 160 mM phosphate, or ascorbate-2-phosphate (AsP). DHA is 95% stable to autoclaving only in water or saline. Storage of DHA solutions at 50 C (to simulate warehouse conditions) showed it to be unstable in water or saline. The pH dependence of DHA breakdown was evaluated; under basic conditions the dimer dendroketose is produced in 8 stereoisomer forms, under acidic conditions methyl glyoxal is the principal product.

Studies are in progress to determine the optimum DHA concentration for red cell 2,3-DPG, with minimal loss of ATP. Preliminary studies indicate 30 mM as best, higher concentrations giving higher 2,3-DPG, but at the expense of red cell ATP concentrations. Two dimers of DHA, dendroketose, and 2-ketogulonic acid have been evaluated in blood and were found to have no efficacy in raising 2,3-DPG. In fact, the latter caused quicker loss of 2,3-DPG and ATP than controls. Two ester derivatives (DHA as the alcohol group) have been synthesized and are being purified to testing in blood. These were made from propionyl chloride and palmitoyl chloride.

#### CONCLUSIONS

DHA is stable in liquid storage in H<sub>2</sub>O or saline at room temperature and could be used in blood storage if kept in a satellite bag at 25 C until the blood is drawn into the bag system. Cost and logistical limitations make the satellite system undesirable, and efforts to find a stable derivative are still underway.

#### RECOMMENDATIONS

Continued evaluation of DHA derivatives is needed for extended liquid storage. Further storage studies at 50 C are needed.

#### PUBLICATIONS

1. LEDFORD, M.E., G.L. MOORE, and T.A. BENSINGER. A comparison of two 2,3-DPG assays. Clin Chem, in press, 1978

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OE 6087	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DES'N INSTR*	9. LEVEL OF SUM	
77 07 19	D. Change	U	U	NA	NL	A. WORK UNIT	
10. NO./CODES*		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		62172A	3S162172A814	00	004		
b. <del>SECONDARY</del>		62772A	3S762772A814	00	004		
c. <del>THIRDARY</del>		CARDS 114 f					
11. TITLE (Precede with Security Classification Code)*							
(U) CPD-Adenine Clinical Trials							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 008800 Life Support							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 01		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
d. NUMBER: Not Applicable				FISCAL YEAR		77	
c. TYPE:				CURRENT		2.0	
e. KIND OF AWARD:				78		2.0	
f. CUM. AMT.						41	
18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
ADDRESS: Presidio of San Francisco, CA 94129				ADDRESS: Department of Surgery			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)			
NAME: Canham, J.F., COL, MC				NAME: Peck, Carl C., LTC, MC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Moore, Gerald L., PhD, DAC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Blood Transfusion; (U) Adenine; (U) CPD;							
(U) Blood Storage; (U) Military Blood Banking							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The final objective of this study, clinical trials of an improved anticoagulant, is Food and Drug Administration licensure, which would permit clinical use of red cells after prolonged liquid storage. Shipment of blood into combat areas necessitates delays between drawing and infusion; the impact of these delays on the quality of red cells infused will be minimized through use of an improved anticoagulant-preservative solution.</p> <p>24. (U) Current red cell liquid storage is limited to 21 days. Data derived through use of an anticoagulant-preservative solution containing citrate-phosphate-dextrose (CPD), adenine, and additional glucose, suggest the storage period can be extended to 35 days, and postinfusion red cell survival would be superior at any given day of storage. Military and civilian investigators, in a cooperative effort, will perform clinical trials under an application for investigational new drug (I.N.D.) held by the container-solution manufacturers. The Blood Research Division, LAIR, is the team leader for this cooperative effort. Data from each study will be collated and analyzed by this division in conjunction with the Department of Information Sciences, LAIR.</p> <p>25. (U) 76 10 - 77 09 Results of a clinical trial using modified CPD (160.9 mM glucose and 2.05 mM adenine) have been completed and the results have been published. Further CPD-adenine clinical trials may be initiated in an attempt to improve the quality of the red cell concentrates after 35 days of storage.</p>							

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation

WORK UNIT NO. 004 CPD-Adenine Clinical Trials

Red cell survival studies in Phase IA of the national cooperative clinical trials of modified CPD-adenine CPD-A<sub>1</sub>: 1.83 g glucose (160.9 mM) and 0.0173 g adenine (2.05 mM) per 63 ml anticoagulant) have been completed, analyzed, and the results published (Transfusion 17:374, 1977). CPD-A<sub>1</sub> appears to be an adequate preservative for 35-day whole blood storage and for 28-day storage of packed red cells (PC). It is only marginally acceptable for 35-day PC preservation. Just prior to FDA approval of the New Drug Application for 35-day blood stored in CPD-A<sub>1</sub>, it was revealed that the plastic bags supplied by one manufacturer (for use in the cooperative clinical trials) were mislabeled as to plastic type (labeled PL-146; true identity was PL-130). At a meeting of FDA and manufacturer representatives with principal investigators from cooperating laboratories, it was agreed to repeat red cell survival studies in a modest number of cases stored in correctly labeled bags (PL-146). Once the studies are completed, licensure should be forthcoming. Plans are being made to evaluate improved CPD-adenine formulations (CPD-A<sub>2</sub>, A<sub>3</sub>: 2.8, 3.2 g glucose (225.2, 257.4 mM) and 0.0346 g adenine (3.03 mM) per 63 ml anticoagulant) which is intended to achieve 42-day storage life for whole blood and red cell concentrates.



## BODY OF REPORT

WORK UNIT NO. 004

CPD-Adenine Clinical Trials

### PROBLEM

Military logistics require extended storage of red cells, particularly to support transfusion requirements in remote areas during hostile activities. Over the past 16 years, work performed by the Blood Research Division at Ft. Knox, Kentucky and by Army contractors has been directed at prolonging shelf-life of red cells for transfusion by fortifying citrate-phosphate-dextrose (CPD) anticoagulant solution with purine bases (adenine, inosine, guanine). It has become apparent from many studies that adenine is effective and accompanied by a low risk of toxicity. However, despite millions of units of blood containing adenine which have been transfused in Europe, extended liquid storage is not available for military use. Clinical trials were needed. Previous civilian efforts to institute clinical trials were not successful for reasons unrelated to the scientific merits of the anticoagulant. With the advent of a national blood policy, it became clear that interface of civilian and military donor resources would be essential to meet sudden large requirements. Thus, general licensing of an adenine-fortified anticoagulant would be necessary if the military were to use it most effectively. Efforts were initiated under this work unit to obtain general Food and Drug Administration (FDA) licensure, with the realization that, even should this not be successful, data would be available to permit use in those unique military settings in which extended liquid blood storage might be essential. If the data suggested lack of safety and efficacy, then further Army funding of adenine blood research would require careful scrutiny.

### RESULTS AND DISCUSSION OF RESULTS

Nine additional 24-hour autologous postinfusion  $^{51}\text{Cr}$ -tagged red cell survival studies of packed red cells (PC) stored for 35 days in CPD-A<sub>1</sub> were performed. These completed the requirements of Phase IA of the IND protocol. The mean percent survivability of the entire 19 PC cases (Table I) was not materially different from that reported for the first 11 cases reported in last year's report ( $71.02 \pm 10.0\%$  [SD]).

A summary of all Phase IA red cell survival results appears in Table I. Whole blood stored for 35 days and PC stored for 28 days exhibit acceptable survivability; i.e., greater than 95% of such units are expected to have survivabilities in excess of 70%. However, PC stored for 35 days in CPD-A<sub>1</sub> yielded only marginally acceptable results. Despite the mean survival of greater than 70%, 45% of such units would be expected to show survivability of less than that.

The Efficacy Panel for Blood and Blood Products of the Bureau of Biologics (BoB), FDA, reviewed the above results in great detail at

TABLE I

Red Cell Survivabilities (CPD-A<sub>1</sub>)

Blood Product	No. Cases	Storage Time (Days)	Survival (%, $\pm$ SD)
Whole Blood (Hct=40)	32	35	80.53 $\pm$ 6.44
Packed Red Cells (Hct=70)	19	35	71.38 $\pm$ 10.30
	8	28	83.97 $\pm$ 6.10

a meeting held 30 September to 1 October 1976. The Panel unanimously recommended that a New Drug Application (NDA) sponsored by one or both of the drug companies supplying anticoagulants and blood bags for the cooperative trial be favorably considered by FDA.

It was recently revealed, however, that blood bags supplied by one manufacturer for use in the cooperative clinical trial of CPD-A<sub>1</sub> had been mislabeled. What was thought to be PL-146 plastic (and labeled as such) was, in fact, PL-130 plastic. A meeting at the BoB (FDA) was held, at which representatives of the BoB (FDA), the manufacturer of PL-130/PL-146 blood bags, Blood Research Division (LAIR), and several laboratories cooperating in the cooperative clinical trials were present. After it was made known that PL-130 plastic blood bags would not be available in the future, the participants at this meeting agreed that additional survival studies of whole blood and PC stored for 35 days in CPD-A<sub>1</sub> within PL-146 (correctly labeled) would be necessary. The Blood Research Division, LAIR, committed itself along with 2 other laboratories to performing the necessary studies (estimated to be approximately 5 whole blood and 10 to 15 red cell concentrate units in total). FDA representatives stated that, after those few remaining survival studies were completed satisfactorily, no substantive issues would remain which might delay favorable consideration of an NDA.

In view of the marginally acceptable survivabilities of PC units stored for 35 days in CPD-A<sub>1</sub>, the participants of the recent BoB (FDA) meeting agreed that improved formulations of modified CPD-adenine should be developed and tested. An increase in the glucose content to 2.8, 3.2 g (225.2, 257.4 mM) and adenine to 0.0346 g (2.05 mM) per 63 ml anticoagulant (CPD-A<sub>2</sub>, A<sub>3</sub>) appears promising. However, some scientific issues remain unresolved (e.g., does increasing glucose concentrations have a deleterious effect on platelet concentrates harvested from whole blood drawn into CPD-A<sub>2</sub>, A<sub>3</sub>?). The Blood Research Division, LAIR, along with other laboratories, agreed to perform necessary investigations on CPD-A<sub>2</sub>, A<sub>3</sub> as a prelude to possible clinical testing. Clinical testing of this improved CPD-adenine preservative would be aimed at developing the capability for 42-day storage life.

### CONCLUSIONS

The licensure effort for CPD-A<sub>1</sub> has been temporarily delayed. Studies required for licensure are underway, and licensure should be forthcoming within 12 to 18 months. An improved CPD-adenine formulation is being developed. This should eventually result in 42-day storage life for whole blood and red cell concentrates.

### RECOMMENDATIONS

1. Additional red cell survival studies should be performed on blood stored in CPD-A<sub>1</sub> for 35 days in PL-146 plastic bags in order to satisfy remaining licensure requirements.
2. In vitro studies of CPD-A<sub>2</sub>, A<sub>3</sub> should be performed as a prelude to in vivo evaluation of this improved CPD-adenine preservative.
3. The Division should participate with the manufacturers in applying for a new drug application, and apply through the Surgeon General's office for the required amendment to the institutional blood bank license held by The Surgeon General to permit the Army, as dictated by military requirements, to use CPD-adenine as formulated in this study.

### PUBLICATIONS

1. ZUCK, T.F., T.A. BENSINGER, C.C. PECK, et al. The in vivo survival of red blood cells stored in modified CPD with adenine. Report of a multi-institutional cooperative effort. Transfusion 17:374, 1977
2. ZUCK, T.F. In: Proceedings of the Workshop on Adenine and Red Cell Preservation (Washington, D.C., 1-2 October 1976), U.S. Department of Health, Education, and Welfare, Bureau of Biologics, Food and Drug Administration, 1976. pp 1-74 to 1-88



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DES'N INSTR <sup>a</sup>	8b. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62172A	3S162172A814		00	005		
b. <del>CONTAMINANT</del>	62772A	3S762772A814		00	005		
c. <del>CONTAMINANT</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Evaluation of DEHP (di-2-ethylhexyl-phthalate) Disposition in Primates (Including Man)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
016800 Toxicology; 012600 Pharmacology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 07		79 01		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER <sup>a</sup> Not Applicable				FISCAL		77	
c. TYPE:				YEAR		1.5	
d. KIND OF AWARD:				CURRENCY		58	
e. AMOUNT:				78		1.0	
f. CUM. AMT.						35	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>a</sup> Letterman Army Institute of Research				NAME <sup>a</sup> Letterman Army Institute of Research			
ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Blood Research Division			
				Department of Surgery			
				Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Canham, J.E., COL, MC				NAME <sup>a</sup> Peck, Carl C., LTC, MC			
TELEPHONE: (415) 561-3600				TELEPHONE: (415) 561-5875			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Odom, Daniel G., CPT, MSC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Toxicity Testing; (U) Polyvinylchloride;							
(U) Di-2-ethylhexyl-phthalate (DEHP); (U) Blood Storage Containers; (U) Plasticizer							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objective is to determine the distribution, biotransformation, and elimination characteristics of Di-2-ethylhexyl-phthalate (DEHP) in primates (including man), as a step towards assessment of the potential for human toxicity of this contaminant in blood stored in polyvinylchloride (PVC) bags. (PVC bags are currently the method of choice, in the military, for transport and storage of blood.)							
24. (U) Following development of a sensitive and specific assay for DEHP and its metabolites, the dependence of DEHP leaching on hematocrit and plastic surface area exposed, time, and temperature will be determined. The pharmacokinetics and metabolism of DEHP will be determined in the monkey and man.							
25. (U) 76 10 - 77 09 The metabolism of DEHP in man has been determined. A study of the disposition kinetics of <sup>13</sup> C-DEHP in man is in a preliminary stage. Mono-2-ethylhexyl-phthalate (MEHP) has been discovered in stored blood. The origin of this DEHP metabolite appears to be in vitro hydrolysis by plasma lipase.							



# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 005 Evaluation of DEHP (Di-2-ethylhexyl-phthalate) Disposition in Primates (Including Man)

The following investigations have been conducted under this Work Unit:

- STUDY NO. 1 DEHP assay development
- STUDY NO. 2 Factors affecting DEHP leaching
- STUDY NO. 5 Pharmacokinetic evaluation of DEHP

Increasing the storage duration for whole blood and packed red cells will improve the logistics of field transfusion therapy. The plasticizer di-2-ethylhexyl-phthalate (DEHP) leaches from polyvinylchloride (PVC) plastic blood bags into the plasma during storage. Concern has been expressed about its potential toxicity in transfusion recipients. Since DEHP leaching from PVC increases approximately linearly with time, prolonged storage increases potential toxicity. To assess properly the increased risk of exposure to DEHP, detailed studies of leaching characteristics, the factors affecting leaching rates, and pharmacokinetic evaluation are essential.

STUDY NO. 1. A combined MEHP (mono-ethylhexyl-phthalate)/DEHP gas chromatographic assay has been developed. The assay simultaneously measures DEHP and MEHP from a single sample and is sensitive, accurate, and precise.

STUDY NO. 2. MEHP has been discovered in plasma of blood stored in plastic bags. It is neither a component of bags nor a natural constituent of blood, but arises de novo during storage; it results from enzymatic hydrolysis of leached DEHP. The accumulation rate of MEHP appears to be affected by the inner surface characteristics of the plastic storage bag.

STUDY NO. 5. Quantitative evaluation of urinary metabolites of DEHP administered to 2 patients via platelet concentrate infusions has yielded preliminary information of the metabolism of DEHP in man. DEHP is metabolized to MEHP and oxidation products of MEHP. These metabolites are excreted mainly in the urine as glucuronide conjugates.

## BODY OF REPORT

WORK UNIT NO. 005

Evaluation of DEHP (Di-2-ethylhexyl-phthalate) Disposition in Primates (Including Man)

STUDY NO. 1

DEHP assay development

### PROBLEM

Prolonging the liquid storage shelf life of whole blood and packed red cells is one of the primary missions of this department. Red cell out-dating poses a major problem in combat medical supply logistics, which could be solved, in part, by the availability of extended storage. During liquid storage of red cells for transfusion, the plasticizer di-2-ethylhexyl-phthalate (DEHP) leaches from the polyvinyl chloride (PVC) plastic storage container into the plasma. The rate of DEHP accumulation in stored plasma is approximately linear with time, and thus, higher concentrations are observed during extended storage intervals. Concern has been expressed regarding the potential toxicity of DEHP in transfusion recipients, although current data of toxic effects are inconclusive. If it is to be recommended that the shelf life of whole blood and packed red cells be extended during combat operations, the potential risks of increasing the dose of DEHP must be evaluated accurately. Under this work unit assay techniques, leaching characteristics, and pharmacokinetics of DEHP are being evaluated.

The discovery of mono-ethylhexyl-phthalate (MEHP) in stored plasma (see Study No. 2 of this work unit) by our collaborating laboratory (Dr. P. Albro, National Institute of Environmental Health Sciences, Research Triangle Park, NC) prompted the incorporation of a plasma assay for MEHP into our previously developed DEHP assay.

### RESULTS AND DISCUSSION OF RESULTS

A combined MEHP/DEHP gas chromatographic assay, with the use of di-n-octal-phthalate (DNOP) as an internal standard, has been developed. Concentrations of these 2 compounds can be simultaneously measured in 1 ml of plasma. Performance characteristics of the assay are shown in Table I.

TABLE I

<u>Characteristic</u>	<u>MEHP</u>	<u>DEHP</u>
Sensitivity	≥12.5 nM/ml	≥12.5 nM/ml
Accuracy <sup>†</sup>	100%	100%
Precision (% C.V.) <sup>††</sup>	±9%	±5%

<sup>†</sup>accuracy is 100% since the internal standard (DNOP) is carried through the entire assay procedure (including preliminary extractions)

<sup>††</sup>applies to concentration range 12.5-300.0 nM/ml

### CONCLUSIONS

The newly developed combined MEHP/DEHP gas chromatographic assay is sensitive, accurate, and precise.

### RECOMMENDATIONS

The new assay should be applied routinely to DEHP leaching and MEHP accumulation studies under this work unit.

### PUBLICATIONS

None

STUDY NO.      2                              Factors affecting DEHP leaching

### PROBLEM

Developing ways to limit human exposure to DEHP (in plasma) requires identification of factors affecting DEHP leaching into plasma from polyvinylchloride (PVC) blood bags. This will permit selection of storage conditions which minimize leaching.

During studies of  $^{14}\text{C}$ -DEHP disposition in the African Green Monkey (Study No. 5, FY 76), small quantities of mono-ethylhexyl-phthalate (MEHP) were discovered in infusion plasmas. Since MEHP is known to be at least as toxic as DEHP, more information regarding the origin of this blood storage contaminant is necessary.

### RESULTS AND DISCUSSION OF RESULTS

MEHP was not found to be a component of plastic blood bags (Fenwal PL-130, McGaw BB-69) nor was it detectable in blood from normal humans. Its origin in plasma of stored blood is from enzymatic hydrolysis of leached DEHP by plasma nonspecific lipase (esterase). This enzymatic activity can be completely destroyed by heating plasma at 60 C for 25 minutes in a water bath. The rate of enzymatic conversion of MEHP from DEHP is more rapid in McGaw BB-69 blood bags than in Fenwal PL-130 bags. We postulate that this disparity arises from surface differences between the 2 plastics. The toxicological significance of MEHP in stored blood is unknown.

### CONCLUSIONS

MEHP arises during blood storage as a result of enzymatic hydrolysis of leached DEHP. The rate of this reaction may be due to surface characteristics of the plastic storage bag.

## RECOMMENDATIONS

Factors affecting the conversion of MEHP from DEHP during storage should be investigated further. Accumulation of DEHP and MEHP should be investigated in current and future blood and blood product preservation systems. Consideration should be given to the study of the toxicological significance of MEHP.

## PUBLICATIONS

None

STUDY NO. 5

Pharmacokinetic evaluation of DEHP

## PROBLEM

The pharmacokinetics and metabolic fate of DEHP in primates has been elucidated in a subhuman primate species (African Green Monkey; see Annual Report FY 76, this work unit), but not in man. In order to assess risks of toxicity from this blood storage contaminant during massive transfusions, the metabolic disposition of DEHP in man must be investigated.

## RESULTS AND DISCUSSION OF RESULTS

DEHP and its metabolites were measured by gas chromatography (GC) in urine from 2 patients receiving DEHP-laden platelet concentrates (Case I: 94.7 mg DEHP infused in 4 hours; Case II: 174.3 mg in 1½ hours). In case I, 57.1% of infused phthalate appeared in urine by 8.5 hours; in Case II, 62.0% was accounted for by 24 hours. Only trace quantities of unchanged DEHP were excreted. Eighty percent of the urinary DEHP metabolites were conjugated to glucuronide. GC-mass spectroscopy revealed the metabolites to be mono-ethylhexyl-phthalate (MEHP) or 8 oxidized derivatives of MEHP. The predominant species was mono(2-ethyl, 3-carboxylpropyl)phthalate (24% of dose, Case II).

## CONCLUSIONS

In 2 patients, intravenous DEHP was extensively metabolized to glucuronide conjugates of MEHP and oxidized MEHP derivatives. These metabolites were rapidly excreted, primarily in the urine.

## RECOMMENDATIONS

A definitive investigation of the disposition of DEHP in man should be undertaken using <sup>13</sup>C-DEHP.



#### PUBLICATIONS

1. PECK, C.C., and T.F. ZUCK. International Forum: What is the toxicological importance of the liberation of phthalates from plastic containers into blood, its components, and derivatives? Vox Sang 34:244, 1978
2. PECK, C.C. Disposition and metabolism of DEHP in primates. In: Transcript of Proceedings, Workshop on Adenine and Red Cell Preservation. Department of Health, Education and Welfare, Bureau of Biologics, Food and Drug Administration, Washington, D.C., 1 Oct 76, pp 1-161
3. PECK, C.C., P.W. ALBRO, D.G. ODOM, and J.R. HASS. Plasticizers in stored blood. In: Microaggregates: Experimental and Clinical Aspects, edited by L. Kozloff. St. Louis, Warren H. Green, Inc. (in press)
4. PECK, C.C., F.J. BAILEY, D. ODOM, H.E. BLATT, and B.B. BARRETT. Plasticizer kinetics in a subhuman primate species. (Abstract) Transfusion 16:526, 1977
5. PECK, C.C., and T.F. ZUCK. DEHP in blood (letter to the editor). Transfusion 17:400, 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 77 07 19	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>a</sup> NA	8. DISSEM INSTRN <sup>a</sup> NL	9a. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3S162172A814	00	006			
b. <del>SECONDARY</del>	62772A	3S762772A814	00	006			
c. <del>THIRDARY</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) The Toxicity of Adenine When Used in Blood Anticoagulant Solution							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 016800 Toxicology; 012600 Pharmacology; 008800 Life Support							
13. START DATE 75 07		14. ESTIMATED COMPLETION DATE 78 06		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT/GRANT a. DATES/EFFECTIVE: b. NUMBER: Not Applicable c. TYPE: d. KIND OF AWARD:				18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 77 78		19. PROFESSIONAL MAN YRS 1.5 0.8	
20. RESPONSIBLE DOD ORGANIZATION NAME: Letterman Army Institute of Research ADDRESS: Presidio of San Francisco, CA 94129 RESPONSIBLE INDIVIDUAL NAME: Canham, J.E., COL, MC TELEPHONE: (415) 561-3600				21. PERFORMING ORGANIZATION NAME: Letterman Army Institute of Research Blood Research Division ADDRESS: Department of Surgery Presidio of San Francisco, CA 94129 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Moore, Gerald L., PhD, DAC TELEPHONE: (415) 561-5875 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Peck, Carl C., LTC, MC			
22. GENERAL USE Foreign Intelligence Not Applicable				POC: DA			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Toxicity Testing; (U) Adenine; (U) Blood Preservation; (U) 2,3 Dioxadenine; (U) Nephrotoxicity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) The 21 day storage limit of blood used to transfuse the wounded soldier can be increased 66 to 100% by the addition of adenine to the preservative. Metabolic and kinetic data will be gathered to define the efficacy and toxicity of adenine when used as part of an anticoagulant solution for the storage of human blood. Investigations will be conducted to determine if 2,8 dioxadenine, a catabolic metabolite of adenine, could accumulate in sufficient quantities in the massively transfused individual to cause toxicity problems.  24. (U) The rate of transport of adenine into red cells during cold storage (whole blood and red cell concentrates) and the rate of conversion to adenine nucleotides will be investigated. The kinetics of adenine catabolic metabolism by xanthine oxidase will be evaluated.  25. (U) 76 10 - 77 09 When whole blood containing 0.25 mM adenine is converted to red cell concentrates for storage, the adenine dose is marginally sufficient. Data indicate improved long-term storage viability by increasing the adenine concentration at time of drawing or by adding 17.3 mg adenine after packing cells. Partitioning of adenine between plasma and red cells has been quantitated; the evidence indicates a higher concentration in red cells with possible membrane binding. Xanthine oxidase kinetics of purines (by using HPLC analysis) indicate incorrect activities are obtained from spectrophotometric analysis of similar systems.							

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 006 The Toxicity of Adenine When Used  
in a Blood Anticoagulant Solution

The following investigations have been conducted under this Work Unit:

STUDY NO. 2 Diffusion of adenine into and out of red blood cells during cold storage

STUDY NO. 3 Adenine metabolism and kinetics in CPD-adenine stored blood

STUDY NO. 2. When units of whole blood drawn into CPD containing 17.3 mg of adenine were stored as red cell concentrate, the plasma adenine concentration was essentially depleted by 21 days in contrast to its persistence through 42 days in whole blood. Moreover, the packed cell ATP levels were significantly lower than whole blood ATP values after 28 days of storage. Addition of 17.3 mg of adenine to blood after packing the cells produced superior ATP concentrations during storage.

STUDY NO. 3. The addition of twofold extra phosphate to stored blood resulted in better ATP maintenance and slightly increased plasma adenine uptake, but this occurred at the expense of red cell 2,3-DPG. Initial studies on the kinetics of xanthine oxidase with hypoxanthine and adenine were evaluated by high pressure liquid chromatography as well as spectrophotometry. The data indicate that erroneous results are obtained by using the standard spectrophotometric procedure. Data have been generated for the development of a mathematical model of red cell metabolism under storage conditions which may yield predictions for optimizing storage conditions.

## BODY OF REPORT

WORK UNIT NO.	006	The Toxicity of Adenine When Used in a Blood Anticoagulant Solution
STUDY NO.	2	Diffusion of adenine into and out of red blood cells during storage
STUDY NO.	3	Adenine metabolism and kinetics in CPD-adenine stored blood

### PROBLEM

Our goal is to gather data on the efficacy and potential toxicity of adenine when it is used as part of an anticoagulant solution for the storage of human blood, and to maximize the beneficial effects of adenine in both whole blood and red cell concentrates (RCC) when it is part of the anticoagulant mix or when it is used in an optional additive system. In addition, we will determine the kinetics of adenine uptake, egress, and metabolism by red cells in these situations, and determine the kinetic limitations of conversion of adenine to its insoluble oxides by xanthine oxidase.

### RESULTS AND DISCUSSION OF RESULTS

When 17.3 mg of adenine is mixed with whole blood, it quickly equilibrates between plasma and red cells to give a net plasma adenine concentration of about 27  $\mu\text{g/ml}$ . During 42 days of 4 C storage, the plasma adenine gradually drops to about 2 to 4  $\mu\text{g/ml}$ ; this reflects its uptake into the red cell and incorporation into the adenine nucleotide pool. The result is increased red cell survivability and elevated red cell ATP concentrations. In this situation, the ratio of adenine to red cells is approximately 90  $\mu\text{g/ml}$  RBCs, depending on the hematocrit (Hct). When the blood is stored as RCC (packed cells), we found that 6.7 mg of the adenine is lost during plasma removal, 10.6 mg is left in the bag of RCC. This reduces the ratio of adenine to red cells to 55  $\mu\text{g/ml}$  RBC. When plasma adenine was monitored during 42 day RCC storage, the concentration started at 27  $\mu\text{g/ml}$  as in whole blood, but approached close to zero by the 21st day. The corresponding red cell ATP concentrations were significantly lower than those in similarly stored whole blood after Day 28. These data are in support of the clinical trials done by Zuck et al. (Work Unit 004) who suggested marginal survivals of RCC after 35 days when starting with an adenine concentration of 0.25 mM in the blood. The addition of 17.3 mg of adenine to the red cells after preparing RCC (i.e., keeping the ratio at 90  $\mu\text{g/ml}$ ) gave superior maintenance of cellular ATP during 42 days of storage.

RCC prepared from CPD adenine blood was also stored with double the CPD concentration of phosphate and/or glucose, but the effects were insignificant on the cellular ATP concentrations. When whole blood units



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(with adenine) were stored with extra phosphate, slightly higher concentrations of ATP were obtained, but controls indicated this was due to  $\text{PO}_4$  stimulation of glucose metabolism. Plasma adenine uptake was somewhat faster, as was predicted from the known activation of phosphoribosyl pyrophosphate synthetase levels by increased  $\text{PO}_4$ . The 2,3-DPG concentrations were decreased by elevated phosphate, thus offsetting the gains made in cellular ATP maintenance.

When 17.3 mg of adenine is added to whole blood, it quickly reaches an equilibrium between plasma and red cells which, in theory, would yield a plasma adenine concentration of 39  $\mu\text{g/ml}$ . The measured value of 27  $\mu\text{g/ml}$  indicated higher red cell concentrations. This was confirmed using  $^{14}\text{C}$ -adenine and chromatography to show a 23% excess of the equilibrium adenine concentration inside the red cells, of which 42% would not egress when the cells were equilibrated with isotonic saline. This suggested a binding of adenine inside the red cell and was confirmed with equilibrium dialysis experiments of adenine with stroma and soluble red cell proteins. Adenine was shown to bind to stroma with a combining affinity of 0.35.

In order to maximize the efficacy of adenine and glucose in blood storage, the development of a mathematical model of the system was initiated. Whole blood drawn in citrate-phosphate, CPD, and CPD-adenine was stored for 63 days and assayed 3 times weekly for adenine, ATP, 2,3-DPG, pH, glucose, and phosphate. Mathematical analysis is now in progress with computer data fitting to differential equations which define the variables in the system. Work was initiated on the development of an inorganic phosphate assay which allows measurement of inorganic phosphate in the presence of organic phosphates such as 2,3-DPG.

Initial attempts were made to define the mixed substrate kinetics of xanthine oxidase. The standard published assays measure the generation of products spectrophotometrically, i.e., uric acid from hypoxanthine and 2,8-dioxyadenine from adenine. When these assays were run in our laboratory, unusual results were seen, and we reassayed each sample by high pressure liquid chromatography. This revealed the appearance of intermediates (xanthine and 8-oxyadenine) during the course of the reaction whose concentrations varied and confounded the spectrophotometric measurement of end products.

#### CONCLUSIONS

When blood containing 0.25 mM adenine is stored as RCC, the available adenine is marginal for adequate ATP levels and survivabilities during 35 to 42 day 4°C storage. This could be corrected by using more adenine (0.40 mM in blood) or by adding the adenine after preparation of RCC. Some binding of adenine to red cells occurs, but this is an asset, if anything (when making RCC). The optimum levels of adenine, and perhaps glucose, have not yet been defined.

### RECOMMENDATIONS

More experimental and mathematical work should be done to define the optimum concentrations of adenine and glucose for extended liquid storage of red cells at a variety of hematocrits.

### PUBLICATIONS

1. MOORE, G.L., and M.E. LEDFORD. The uptake and egress of adenine from human red blood cells. *Transfusion* 17:38, 1977
2. PECK, C.C., F.J. BAILEY, and G.L. MOORE. Enhanced solubility of 2,8 dihydroxyadenine (DOA) in human urine. *Transfusion* 17:383, 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DMS'N INSTR'M	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES: <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3S162172A814	00	007			
b. <del>SECONDARY</del>	62772A	3S762772A814	00	007			
c. <del>THIRDARY</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Development of a Rapid System for Assessing Blood Anticoagulant-Nutrient Preservatives							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology; 003500 Clin Medicine; 009800 Medical and Hosp Equip; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: EXPIRATION:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: Not Applicable				FISCAL YEAR 77		0.5	
c. TYPE: d. AMOUNT:				CURRENT YEAR 78		53	
e. KIND OF AWARD: f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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ADDRESS: Presidio of San Francisco, CA 94129				ADDRESS: Div of Combat and Experimental Surg Department of Surgery Presidio of San Francisco, CA 94129			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME: Bensinger, Thomas A., LTC, MC			
				NAME: POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rapid Screening Techniques; (U) Blood Preservation; (U) Temperature; (U) Blood Respiratory Function							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objectives are to devise an accelerated method for testing anticoagulant-nutrient preservatives by using elevated temperatures; to determine if rates of change of the observed parameters during storage are predictable on a chemical-kinetic basis; and to apply such techniques to rapid screening of substances and techniques potentially beneficial to preservation of blood and the therapeutic effectiveness of this material for treatment of battlefield injuries.							
24. (U) Results have shown that the rates of change of functionally significant substances, such as H <sup>+</sup> , 2,3 diphosphoglycerate (2,3-DPG), and adenosine triphosphate (ATP), in blood stored at 4 C can be predicted from observations at 37 C. As rates of change are often 30-fold greater than at 4 C, observations at 37 C of the effects of experimental anticoagulant-nutrient preservatives on blood storage can be condensed to about 24 hours compared to 35 or so days required at 4 C. This high temperature approach to such screening will be continued and extended to (a) optimize the composition of preservative media, (b) identify factors altering the collection and storage lesion, and (c) understand differences in individual donors with respect to blood storageability.							
25. (U) 76 10 - 77 09 Data were obtained on human blood stored in citrate-phosphate-dextrose at temperatures between 0 and 8 C to test the hypothesis (see DD form 1498 dated 76 10 01) that small changes in storage temperature (between 0 and 8 C) result in large biochemical effects in stored blood. Storage at 8 C rather than 4 C was detrimental to the quality of stored blood in terms of H ion accumulation and 2,3-DPG depletion. ATP is enhanced at one week of storage at 8 C but depletes rapidly thereafter. Storage at 0 C did not yield expected improvements in organic phosphate retention; fixed acid accumulation was lower than at 4 C. The differences noted are accentuated by prolonged (4-6 weeks) storage.							

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DD FORM 1498

1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE



# ABSTRACT

PROJECT NO. 3S762772A814 Combat Trauma and Resuscitation  
WORK UNIT NO. 007 Development of a Rapid System for  
Assessing Blood Anticoagulant-Nutrient  
Preservatives

Measurements were made of  $H^+$ , 2,3-DPG, and ATP in CPD-preserved human blood at 0, 4, and 8 C. Observations at 4 and 8 C were consistent with those reported previously at 37, 24, 14, and 4 C, i.e., they revealed a predictable relation between storage temperature and the rates of change of the three chemical constituents which were measured. Blood stored at 0 C did not show the expected decrease in rate of change of the measured constituents. In general, the results at this temperature were the same as those at 4 C. On the basis of these in vitro measurements, it is concluded that: 1) the optimum liquid storage temperature of blood preserved in CPD is between 0 and 4 C; 2) small temperature increases above this range are significantly detrimental to the quality of CPD-stored blood; 3) between 37 and 4 C, temperature has a predictable effect on changes of  $H^+$ , 2,3-DPG, and ATP in blood preserved in CPD, i.e. the changes proceed approximately 30 times faster at 37 C than at 4 C.

## BODY OF REPORT

WORK UNIT NO. 007

Development of a Rapid System for  
Assessing Blood Anticoagulant-Nutrient  
Preservatives

### PROBLEM

The supply and quality of liquid stored blood used for transfusion purposes in wounded soldiers can be seriously limited by the exigencies of warfare. Therefore, there is a unique military requirement for prolonging the present 21-day storage limit for preserved liquid blood and improving the quality of this product. Most experiments designed to improve preserved liquid blood generally are performed at 4 C, presumably on the pragmatic ground that this temperature normally is used for liquid blood storage. For several reasons, however, this pervasive experimental approach may be undesirable. In the first place, the practice tends to perpetuate the notion that 4 C is the optimum temperature for blood storage; actually, this idea has not received extensive experimental inquiry. Furthermore, individual experiments at 4 C usually require prolonged periods of time (up to 6 weeks or more) to complete. Because of the variability of individual blood donors and the multiple permutations in storage conditions and preservation media that may require testing, satisfactory experimental confidence in a new media or preservation idea can be slow in developing. Finally, phenomena important to the full understanding of the collection and storage lesions may be suppressed or imperfectly developed at 4 C. Therefore, the objective of this work unit is to devise accelerated means for evaluating anticoagulant-nutrient preservative solutions (ANPS) and other variables affecting red cell storage in order (1) to economize on valuable technical resources, (2) to provide alternative theoretical approaches to understanding the deterioration of red cells during storage, (3) to develop a scientific basis for determining the optimum storage temperature of blood. The initial approach to this problem has been to study the effect of temperature on blood storage. The initial working hypothesis to be tested is whether or not the rates of change in  $H^+$ , 2,3-DPG, ATP, and other factors affecting the quality of stored blood are altered by temperature in accordance with simple and predictable thermokinetic considerations.

### RESULTS AND DISCUSSION OF RESULTS

Blood from a pair of normal human donors was collected in CPD using standard techniques and equal portions of each donor's blood was stored at 0, 4, and 8 C. Immediately after withdrawal and at weekly intervals thereafter for a period of 6 weeks, observations were made of  $P_{50}$  (7.4), heme-heme interaction, pH (37 C),  $P_{O_2}$ ,  $P_{CO_2}$ , 2,3-DPG, ATP, and glucose. Five separate donor pairs have been observed during this reporting period.

Comparing the mean rates of change in  $H^+$ , 2,3-DPG, and ATP observed at 8 C with those obtained at 4 C, the 8 C rates proceeded, in each instance, at about twice the rate found at 4 C. This doubling of the rate of change in these constituents for this small temperature interval was consistent with predictions based on previous results over a much wider temperature span (37 to 4 C), and supports the hypothesis that these changes are amenable to thermokinetic (Arrhenius) analysis. Practically speaking, it appears possible to study blood storage at high temperatures and extrapolate these results to 4 C. The advantage of this approach resides in the fact that changes requiring 5 or 6 weeks at 4 C are completed in 1 or 2 days at 37 C.

When results at 0 C were compared to those at 4 C, no significant differences in the mean rates of change in  $H^+$ , 2,3-DPG, or ATP were found.  $PCO_2$ , however, was consistently higher at 0 C than at 4 C. Consequently, when pH values (and  $H^+$  ion) were corrected to a constant  $PCO_2$  (40 mm Hg), the rate of accumulation of fixed acid (negative base excess) was positively correlated with temperature; at 0 C, this accumulation rate was approximately one-third as rapid as at 8 C and two-thirds the rate found at 4 C.

Storage of blood at 8 C favors retention of ATP during the first week of storage; in fact, higher temperatures have frequently been found to stimulate the generation of ATP during the early storage period, even though subsequent losses are at a faster rate than those at lower temperatures. Because ATP has been found to be related to in vivo survivability of erythrocytes, this biphasic response of ATP to temperature may be important to understanding the collection and storage lesions.

One of the more noteworthy features of the results obtained so far has been the marked degree of variation found in the rates of change of the various factors in the individual donors that have been measured. This aspect of blood preservation has received relatively little attention, probably because of the difficulties of accumulating sufficient information from which to construct viable hypotheses. Many questions that arise from the existence of these individual differences can be effectively pursued at elevated temperatures (37 C).

#### CONCLUSIONS

High temperature observation of stored blood appears to be an effective and economic means of assessing ANPS and studying the effects of blood storage conditions. The relative effect of storage on  $H^+$ , 2,3-DPG, and ATP which are deemed important to the clinical effectiveness of transfused blood, is consistently maintained at all temperatures investigated between 37 and 0 C. High temperatures may unmask phenomena (ATP generation, for instance) not always noted in blood stored at 4 C. Small increases in storage temperature above 4 C significantly degrade the quality of stored blood in terms of  $H^+$ , 2,3-DPG, and ATP (after the first week) levels. Contrary to expectation, 0 C storage did not appear

superior (on the basis of the direct chemical measurements) to 4 C storage with the possible exception regarding fixed acid accumulation.

#### RECOMMENDATIONS

These results should be extended to other ANP solutions. High temperature observations to test the effect of dissolved gases (especially CO<sub>2</sub>) and pH need to be completed.

In vivo erythrocyte survival of blood stored at 0 C should be tested and compared to 4 C storage.

Massive transfusions of blood stored at 0 C may be more effective for fluid replacement therapy of shock than 4 C stored blood because of the diminished acid load presented to the organism. This hypothesis should be tested in an appropriate experimental animal model.

#### PUBLICATIONS

1. NEVILLE, J.R., T.A. BENSINGER, and T.F. ZUCK. Effect of temperature on blood storage. (Abstract) Transfusion 16:517, 1977
2. NEVILLE, J.R. Erythrocyte age and shape of the oxygen dissociation curve. (Abstract) Proc Intl Union Physiol Sci XIII, 1977, p 548



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION <sup>a</sup>	2 DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6305	77 10 01	DD-DR&E(AK)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY <sup>a</sup>	6 WORK SECURITY <sup>a</sup>	7 REGRADING <sup>a</sup>	8A DDB'S INSTR'N	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
77 07 19	D, Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10 NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62172A	3S162172A814	00	008			
B. ODBT NUMBER TO DB	62772A	3S762772A814	00	008			
C. ODBT NUMBER TO DB	CARDS 114 f						
11 TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Anesthetic Management and Perioperative Care of the Acutely Wounded Soldier							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008800 Life Support; 009800 Medical and Hospital Equipment; 012900 Physiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		77	
C. TYPE				CURRENT		1.0	
D. KIND OF AWARD				78		0.6	
E. CUM. AMT.						65	
F. CUM. AMT.						93	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
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				SOCIAL SECURITY ACCOUNT NUMBER			
22 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Applicable				NAME: Moores, William Y., LTC, MC			
				NAME:			
				POC: DA			
23 KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>							
(U)Anesthesia; (U)Combat Injury; (U)Perioperative Care; (U)Respiratory Physiology; (U)Cardiovascular Physiology; (U)Anesthesia-related Equipment							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Because their administration is extremely hazardous in subjects with acute hypovolemia, anesthetic agents are avoided in injured civilians prior to replacement of blood volume. However, timely combat injury care in field settings frequently requires anesthetic administration to soldiers with incompletely replaced blood loss. The objective of this work unit is to establish physiologic data on the effects of anesthesia during acute hypovolemia. These data will permit appropriate selection of premedications, anesthetic agents and adjuncts, and anesthetic procedures for field use. Requirements for anesthetic-related equipment for field use also must be identified, and available equipment evaluated.							
24. (U) The effects of premedications, anesthetic agents, anesthetic adjuncts during hypovolemia, with and without volume replacement, and their interactions of cardiopulmonary function will be studied in dogs and goats. The influence of these factors on the cardiovascular system will also be studied in goats and swine. Attempts will be made to determine the applicability of the data from these studies to the combat-injured soldier.							
25. (U) 76 10 - 77 09 Chronic dogs have been prepared with splenectomy, carotid loops, and tracheostomies. Several anesthetic agents are currently being investigated for their effect on the cardiorespiratory system of dogs during hypovolemia and normovolemia, with and without hypoxia. The cardiopulmonary bypass model in swine has been established. Anesthetic agents are currently being investigated for their effect on myocardial contractility. Goats have been procured and surgical preparation is in progress.							

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 008 Anesthetic Management and Peri-operative Care of the Acutely Wounded Soldier

The following investigations have been conducted under this Work Unit:

- STUDY NO. 1 Selection of anesthetic agents for administration to the acutely wounded soldier
- STUDY NO. 2 Evaluation of myocardial performance during anesthesia
- STUDY NO. 3 Evaluation of tri-service experimental portable volume respirator

STUDY NO. 1. It is not clear which of the available general anesthetics most closely maintain normal physiological function during moderate blood loss. We therefore have embarked upon a series of experiments to provide data comparing several anesthetic agents for their ability to preserve physiological function during moderate hypovolemia. In chronically maintained splenectomized dogs who had been provided with chronic tracheostomies and exteriorized carotid arteries, we have evaluated cardiorespiratory function during graded hypovolemia. The dogs were anesthetized with halothane, which was maintained at an end tidal concentration of 1.0%; step-wise reductions of 10, 20, and 30% of each dog's blood volume were successively made. Progressive hemorrhage to 30% blood loss caused no significant changes in tidal volume, frequency, or minute ventilation. However, significant reductions in mean arterial blood pressure, mean pulmonary arterial blood pressure, cardiac output, stroke volume, left ventricular work, and oxygen transport occurred. Oxygen consumption and base excess did not change with progressive hemorrhage. The lack of change of oxygen consumption and base excess with progressive hypovolemia implies that the tissues' oxygen demands were satisfied, and that anaerobic metabolism did not increase from normovolemia to 30% oligemia. With progressive hypovolemia during halothane anesthesia, end-tidal to arterial oxygen difference increased. This was probably a result of severely disturbed pulmonary ventilation to perfusion relationships caused by severe pulmonary arterial hypotension.

STUDY NO. 2. Myocardial effects of anesthetic agents will be evaluated in the right heart pulmonary bypass swine model. The model has been perfected, and pilot studies performed.

STUDY NO. 3. Through the U.S. Naval Medical Research and Development Command, this laboratory has received for evaluation for the U.S. Army

Medical Research and Development Command a prototype model of the tri-service experimental portable volume control respiratory. The ventilator is being evaluated for compliance with its specifications as well as for compliance with the American National Standard for Breathing Machines for Medical Use (ANSI Z79.7-1976). Bench testing is currently in progress.

## BODY OF REPORT

WORK UNIT NO.	008	Anesthetic Management and Peri-operative Care of the Acutely Wounded Soldier
STUDY NO.	1	Selection of anesthetic agents for administration to the acutely wounded soldier

### PROBLEM

The physiological effects of many of the currently used anesthetic agents are known in great detail in both laboratory animals and humans. However, little information is available regarding the physiological effects of these agents during states of deranged physiology. This is especially true regarding the effect of anesthetic agents during the hypovolemic state. This lack of knowledge is most likely the result of usual civilian practice of restoring blood volume to normal prior to induction of anesthesia. In the vast majority of civilian injuries, this is not only desirable but possible. Unfortunately, the nature and extent of many military injuries require induction and maintenance of anesthesia in an acutely hypovolemic soldier in order that a surgical procedure may be performed to prevent further blood loss and enable restoration of blood volume. The choice of anesthetic agents in this situation, at the current time, can only be based upon anecdotal information, personal preferences, and experience of the anesthesiologist.

Therefore, we have embarked upon a series of experiments to provide data by which the physiological effects of several anesthetic agents during moderate hypovolemia may be compared.

### RESULTS AND DISCUSSION OF RESULTS

Five splenectomized dogs with chronic tracheostomies and exteriorized carotid arteries have been studied during halothane anesthesia. Without premedication, the dogs were anesthetized with halothane, which was maintained at an end tidal concentration of 1.0%. Venous, carotid arterial, and thermodilution pulmonic arterial catheters were placed percutaneously, and cardiorespiratory measurements made. Each animal was bled in a step-wise fashion by removal of 10, 20, and 30% of his or her blood volume, and the measurements repeated at each level of oligemia. Ventilation was measured by a Wedge Spirometer attached to the circle breathing system through a bag-in-a-box. Systemic and pulmonic arterial blood pressures were measured by transducers, and cardiac output by a thermodilution technique in which an analog computer was utilized. The animals were allowed to breath spontaneously at all times. End tidal  $P_{O_2}$  was controlled so as to maintain arterial  $P_{O_2}$  at 100 torr.



Hypovolemia to 30% blood loss caused no significant change in respiratory frequency, tidal volume, or minute ventilation.

With progression from normovolemia to 30% hypovolemia, there were significant changes in systemic mean blood pressure ( $95.6 \pm 3.4$  to  $67.4 \pm 5.6$  torr), mean pulmonary arterial pressure ( $10.1 \pm 2.4$  to  $2.0 \pm 3.2$  torr), cardiac output ( $115.8 \pm 17.3$  to  $73.3 \pm 13.3$  ml/min/kg), stroke volume ( $1.12 \pm 0.11$  to  $0.60 \pm 0.11$  ml/kg), left ventricular minute work ( $343.2 \pm 48.8$  to  $156.0 \pm 28.1$  g meters/min), and oxygen transport ( $19.6 \pm 3.2$  to  $10.7 \pm 2.1$  ml  $O_2$ /min/kg). No changes were noted in oxygen consumption ( $4.10 \pm 0.22$  to  $4.04 \pm 0.31$  ml/min/kg) or base excess ( $-3.3 \pm 0.8$  to  $-3.5 \pm 1.2$  mEq/l). End-tidal to arterial oxygen tension difference increased during progressive hypovolemia ( $36.8 \pm 7.2$  to  $86.4 \pm 31.0$  torr). No changes were noted in either arterial pH ( $7.337 \pm 0.009$  to  $7.312 \pm 0.014$ ) or arterial  $PCO_2$  ( $41.8 \pm 1.3$  to  $40.6 \pm 2.2$ ).

In the operating room, anesthesiologists can measure arterial blood pressure and heart rate, and observe changes in respiratory frequency and tidal volume. Hypovolemia during halothane anesthesia does not cause changes in observed respiratory variables but does cause a dramatic decrease in arterial blood pressure. Ordinarily, hypotension is to be avoided for fear of detrimental decreases in tissue oxygenation. Although hemorrhage during halothane anesthesia caused a decline in cardiac output, arterial blood pressure, and oxygen transport, total body oxygen consumption did not change, nor was base excess altered. We conclude that, despite the fall in oxygen transport, the lack of change in oxygen consumption and base excess with progressive hypovolemia imply that the tissues' oxygen demands were satisfied, and that anaerobic metabolism did not increase from normovolemia to 30% oligemia.

Although tissues seemed to fare quite well during hypovolemia to 30% during halothane anesthesia, this was not true for gas exchange. The increasing difference between end-tidal and arterial  $P_{O_2}$  with progressive hemorrhage during halothane anesthesia was probably a result of severely disturbed pulmonary ventilation to perfusion relationships caused by severe pulmonary arterial hypotension, because  $ETaDO_2$  returned to its original value with the return of shed blood.

#### CONCLUSIONS

Oligemia to 30% blood loss during halothane anesthesia results in decreased systemic and pulmonic arterial blood pressures, cardiac output, stroke work, minute work, and oxygen transport.  $ETaDO_2$  is increased probably as a result of altered  $V/Q$ . Respiratory frequency, tidal volume, minute volume,  $Vo_2$ , base excess, pH and  $PCO_2$  do not change. The lack of change of  $Vo_2$  and base excess indicate that anaerobic metabolism does not increase with 30% hemorrhage during halothane anesthesia.

### RECOMMENDATIONS

Recommendation for choice of anesthetic agent given to an injured soldier cannot be made until the effects of other anesthetic agents are compared with the effects of halothane. Therefore, it is recommended that (1) further physiological studies be carried out to assess physiological effects of halothane during graded hypovolemia; (2) other anesthetic agents be similarly investigated so that their effects may be compared with the effects of halothane and (3) offer information to enable anesthesiologists to make a more rational choice regarding the proper anesthetic agent for utilization in the anesthetization of the acutely wounded soldier.

### PUBLICATIONS

None

STUDY NO. 2

Evaluation of myocardial performance during anesthesia

### PROBLEM

Although it is generally felt that anesthetic agents are myocardial depressants, firm physiological data, with all appropriate variables controlled, are not available. We have therefore undertaken a series of experiments to provide such data.

The swine right heart pulmonary bypass model will be utilized. This model has been perfected at LAIR, and pilot experiments have been successfully carried out.

### RESULTS AND DISCUSSION OF RESULTS

None

### CONCLUSIONS

None

### RECOMMENDATIONS

None

### PUBLICATIONS

None

STUDY NO. 3

Evaluation of tri-service experimental  
portable volume respirator

PROBLEM

All 3 military uniformed services have recognized the need for a portable volume controlled ventilator capable of ventilating injured military personnel in the field, as well as during any one of the many modes of transportation and evacuation of injured personnel as well as in the hospital environment. Currently, commercially available ventilators cannot meet these requirements. As a result, primarily through the Office of Naval Research, General Electric Re-entry and Environmental Systems Division has produced an experimental ventilator designed to meet these needs. Twelve prototypes have been produced. This prototype now requires full laboratory and clinical testing prior to acceptance by the 3 services for production and deployment.

RESULTS AND DISCUSSION OF RESULTS

This laboratory received a prototype of this experimental ventilator. As a result of multiple electrical problems, the initial model was returned to the contractor, repaired, and returned to this laboratory. Bench testing is currently underway to judge whether or not this experimental ventilator meets all specifications of the contract as well as the requirements of the American National Standard for Breathing Machines for Medical Use as promulgated by the American National Standards Institute (Z79.7-1976).

CONCLUSIONS

None

RECOMMENDATIONS

Testing and evaluation of this experimental ventilator be continued.

PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OE 6306	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DOWN INSTR <sup>a</sup>	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 07 19	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62172A	3S162172A814		00	009		
b. SECONDARY	62772A	3S762772A814		00	009		
c. TERTIARY	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Metabolic Support Following Combat Injury							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 Clinical Medicine; 008800 Life Support; 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 10		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER: Not Applicable				FISCAL YEAR		89	
c. TYPE:				CURRENT			
d. KIND OF AWARD:				78		2.5	
e. AMOUNT:						123	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Letterman Army Institute of Research				NAME: Letterman Army Institute of Research			
ADDRESS: Presidio of San Francisco, CA 94129				ADDRESS: Division of Surgical Metabolism			
				Department of Surgery			
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				SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Applicable				NAME: Gourlay, Stuart J., LTC, MC			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>							
(U) Body Compositional Change; (U) Wound Healing;							
(U) Military Trauma; (U) Parenteral Nutrition; (U) Animal Model							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Combat injuries produce a uniquely severe form of stress to normal metabolism. Within days following significant military injury, 21% of body muscle mass will have been lost. Improved techniques of metabolic support may minimize this deleterious body compositional change, maximize wound healing, and reduce morbidity. The objective of these studies is to develop optimal post-traumatic metabolic support techniques and formulae to facilitate rapid return to duty.							
24. (U) An in vitro perfused rodent hind limb, wounded to simulate combat injury, has been prepared. In this preparation, we will (a) study the effects of post-trauma hormonal milieu on normal and wounded muscle substrate utilization, (b) design a post-traumatic regimen which can be deployed in field settings, (c) test formulae in higher animals, and (d) investigate formulae in traumatized man.							
25. (U) 76 10 - 77 09 Three sections have been developed under the work unit during this period: (1) an in vitro perfused rodent hind limb model, (2) a chronic rhesus monkey intravenous nutrition model, and (3) an effective clinical nutritional support service. The perfused hind limb model has been evaluated for physiological stability and hormone sensitivity, and data collection are underway. In-house substrate analyses have been developed for lactate, pyruvate, glucose, urea, ATP, ADP, AMP, creatine phosphate, glycerol, FFA, acetoacetate, $\beta$ -hydroxybutyrate, DNA, Kjeldahl nitrogen and gas-liquid chromatography for plasma fatty acids. The rhesus model has been established to evaluate intravenous solutions to minimize body compositional changes following trauma. Data are currently being gathered which will delineate methods to improve efficiency of protein utilization in combat injured man. The nutritional support service has cared for 134 patients requiring intravenous support during this period (over 3500 patient days). These critically ill patients provide a useful data base for evaluation of the practicality of implementation and safe monitoring of intravenous nutritional support under combat conditions.							



# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 009 Metabolic Support Following Combat Injury

The following investigations are being conducted under this Work Unit:

STUDY NO. 1 Metabolic support following combat injury

STUDY NO. 2 Effects of the ratio of carbohydrate calories to protein supplied intravenously in minimizing body compositional loss following combat injury

STUDY NO. 3 Effects of surgical dressings and procedures on skin bacteria surrounding venous catheters

STUDY NO. 1. A model has been developed in 180-200 g male rats which permits isolation of skeletal muscle metabolism apart from the remainder of the animal. As a consequence, the influence of neurohumeral control and substrate supply on skeletal muscle metabolism can be carefully controlled. Initial evaluation of the preparation involved determination of the presence of adequate perfusion and normal hormonal sensitivity.

Adequate tissue oxygenation was demonstrated by examination of oxygen consumption and tissue adenine nucleotide and phosphocreatine concentrations following perfusion, which were found to agree with *in vivo* values (mmole/g wet wt,  $\bar{X} \pm \text{SEM}$ ). (ATP  $5.77 \pm 0.24$ , ADP  $2.21 \pm 0.10$ , PCr  $15.85 \pm 0.70$ ,  $\text{O}_2$  consumption  $9 \mu\text{mole/min/100 g BW}$ ). Lactate production of  $1.02 \pm 0.17 \mu\text{mole/min/100 g BW}$  compared with published values of  $1.16 \mu\text{mole/min/100 g BW}$  by other investigators. Hormonal sensitivity was demonstrated by increased glucose clearance at 25  $\mu\text{units/ml}$  and further statistically significant increases in clearance at 100, 500, and 1,000  $\mu\text{units/ml}$ . As a consequence of these preliminary data, the isolated perfused rat hindquarter appeared to offer an acceptable experimental model in which to study hormone-substrate interrelationships in muscle and specifically to investigate the effect of the post-trauma hormonal milieu on substrate utilization in muscle.

STUDY NO. 2. Four rhesus monkeys (*Macaca mulatta*) were chaired to allow continuous intravenous infusion of nutrient solutions designed to replace the monkey's daily dietary regimen. Initial problems with catheter placement and dietary requirements were encountered, requiring extensive investigation of caloric, protein, and micronutrient requirements of chaired versus unrestrained monkeys, and modifications of catheter placement. These investigations are concluding at present, and it is felt that the chaired Rhesus monkey model will prove invaluable in evaluating manipulations in hormone and substrate supply in decreasing body composition loss following trauma.

STUDY NO. 3. This protocol is currently undergoing LAIR review. The proposal is designed to determine objectively the optimal techniques for central venous catheter dressing care. As a consequence of this study, a safe, effective, manpower-efficient method should be developed to protect the combat injured from sepsis secondary to central venous catheterization.

## BODY OF REPORT

WORK UNIT NO. 009 Metabolic Support Following Combat Injury  
STUDY NO. 1 Metabolic support following combat injury

### PROBLEM

Combat injured man presents the most pronounced example of stress metabolism. The human metabolic response to this type of trauma is one of extreme catabolism which often results in body compositional losses of over 1 kg (2.2 lb) daily. These compositional changes reflect losses of stored carbohydrate (glycogen), triglycerides (subcutaneous fat), and muscle mass, which are rapidly utilized for energy by the altered post-trauma metabolism. It has been shown with severe injury that the loss of body nitrogen is accelerated from 3 to 5 g per day to a range of 15-30 g (450-900 g muscle) per day for several days. Caloric supply during this period will spare body fat, but has little effect on loss of body nitrogen.

Body fat is considered an efficient form of stored calories, and its utilization for this end during periods of high caloric requirements is not generally considered detrimental. However, muscle is an important structural element, and as a consequence, relatively small losses of muscle mass result in significant alterations in total body function. Loss of limb muscle mass leads to marked weakness and impaired mobility. Loss of intercostal and diaphragmatic muscle mass leads to inability to clear bronchial secretions and may result in pneumonia and death.

The interrelationship between the general biochemistry of injury and convalescence and the local changes of wound healing has not been investigated in detail. Early wound healing also occurs during a period of negative energy balance, i.e., general catabolism. There is clearly a very high biological priority of the wound in the early days and weeks after injury. This favored biological priority, however, is transient. One to two weeks following trauma, there is a prolonged phase of protein synthesis and lipogenesis to restore tissue mass to normal. At this point, if intake of foodstuffs can be resumed, wound healing moves normally toward completion. If, however, there is prolonged starvation of continued catabolism, the wound must begin to compete with other tissues for substrate, and wound healing begins to suffer severely.

The objective of this investigation is to develop a specific therapeutic regimen for post-traumatic metabolic support that will facilitate the rate of wound healing while minimizing body compositional changes. The steps necessary to accomplish this objective are as follows: (1) investigation of the effect of the post-trauma hormonal milieu on substrate utilization by normal muscle; (2) investigation

of substrate utilization by wounded muscle and the alterations in this utilization by the post-trauma hormonal abnormalities; (3) design of a post-trauma therapeutic regimen based on steps 1 and 2 that will maximize the rate of wound healing while minimizing muscle compositional changes; (4) investigation of this formula in traumatized dogs and non-human primates; (5) investigation of this formula in traumatized man; and (6) modification of this formula as necessary for feasibility of use under combat conditions.

#### RESULTS AND DISCUSSION OF RESULTS

The first phase of this study involved development of the experimental model to study muscle metabolism *in situ*. The isolated perfused rat hindquarter technique was developed and evaluated for viability and effect of insulin on glucose clearance.

The preparation maintained constant glucose uptake and oxygen consumption with minimal lactate production for 80 minutes of perfusion. Adequacy of tissue perfusion was shown by tissue concentrations of adenine nucleotides and phosphocreatine comparable to *in vivo* levels (ATP  $5.77 \pm 0.24$ , ADP  $2.2 \pm 0.10$ , AMP  $0.81 \pm 0.22$ , and phosphocreatine  $15.85 \pm 0.70$   $\mu$ moles/g wet muscle). In addition, lactate production was minimal ( $1.02 \pm 0.17$   $\mu$ moles/min/100 g BW) and the preparation consistently maintained a venous perfusate lactate/pyruvate ratio of  $\leq 10/1$ .

The response of glucose clearance to increasing perfusate insulin concentrations was evaluated. Insulin doses of 0, 25, 100, 500, and 1,000  $\mu$ units/ml produced changes in glucose clearance of  $0.13 \pm 0.01$ ,  $0.16 \pm 0.03$ ,  $0.17 \pm 0.02$ ,  $0.32 \pm 0.02$  and  $0.44 \pm 0.03$  ml/min/100 g BW, respectively. Although in this study an insufficient number of animals (3) were done at the 25  $\mu$ unit/ml insulin concentration to prove a statistically significant insulin response, the preparation is obviously extremely insulin sensitive. In addition, previous studies with this preparation have shown a significant increase in glucose clearance at the 25  $\mu$ unit/ml concentration.

To investigate the changes in substrate utilization under the effect of the post-trauma hormonal milieu, analyses for substrate concentrations were necessary. As a consequence, assays for lactate, pyruvate, glucose, urea, ATP, ADP, AMP, phosphocreatine, glycerol, free fatty acids, acetoacetate,  $\beta$ -hydroxybutyrate, Kjeldahl nitrogen, and gas liquid chromatographic separation of individual fatty acids have been established in this period.

#### CONCLUSIONS

A viable hormonally sensitive *in vitro* preparation has been established to study the metabolic response of skeletal muscle to the post-trauma hormonal milieu. Methods for measuring perfusate concentrations of various substrates have been established to allow investigation of hormone-substrate interrelationships.



## RECOMMENDATIONS

With completion of preliminary evaluation of the in vitro model and with established methods for substrate analysis, it is recommended that the experiments outlined in the initial protocol be conducted.

## PUBLICATIONS

1. CALDWELL, M.D., J.H. EXTON, and W.W. LACY. Effect of glucocorticoids on substrate utilization by muscle. (Abstract) Fed Proc 36:527, 1977

STUDY NO. 2

Effects of the ratio of carbohydrate calories to protein supplied intravenously in minimizing body compositional loss following combat injury

## PROBLEM

Tissue catabolism following the stress of trauma, sepsis, and major surgery is a common and well established phenomenon. The consequences stemming from this catabolic state are protean and include generalized muscle wasting, impaired wound healing, and impaired immune response. Deterioration in any of these critical factors compounds any clinical problem leading to further complications and potentiation of the catabolic state.

The catabolic response to trauma with resultant changes in body composition is thought to be secondary to a complex interrelationship between the post-trauma hormonal milieu and exogenous substrate supply. In recent years, due to the advent of total parenteral nutrition, it has become possible to reduce and in some cases reverse this catabolic cycle by vigorous intravenous nutritional support. It has been proposed that an optimal relationship exists between endogenous hormonal flux and exogenous substrate provision that will minimize body compositional change following trauma. This has yet to be determined. In addition, when intravenous nutrition is used under combat conditions, a decrease in the amount of intravenous protein necessary for each individual would result in substantial savings in transport space and weight. This also facilitates our logistic capabilities for providing this type of therapy.

## RESULTS AND DISCUSSION OF RESULTS

The first phase of this study involved establishment of the experimental model. This involved chronically-maintained male rhesus monkeys with indwelling central venous catheters. Two specific problems were encountered: (1) available data regarding the nutrient requirements for 7 kg monkeys were found to be in error for chaired monkeys. When nutrients were supplied intravenously to meet reported requirements,

the monkeys uniformly lost 0.5 to 1.0 kg body weight and had a marked loss of subcutaneous fat. (2) Broviac silastic catheters were placed in the internal jugular vein and guided through a subcutaneous tunnel to exit over the occipital region. This site of exit was found to provoke chronic head-shaking with chronic catheter-cutaneous junction irritation and eventual catheter sepsis in 3 monkeys.

As a consequence of the above problems, a new satisfactory method for catheter placement has been devised and a detailed study of nutrient intake in chaired monkeys has been completed. The latter study has revealed that chaired monkeys require a 75 to 80% increase in caloric intake to maintain stable body weight and nitrogen equilibrium. As a consequence, maintenance intravenous nutrient solutions have been designed for chaired monkeys to allow progression of this protocol.

#### CONCLUSIONS

A chronically maintained monkey model on complex intravenous nutrition is feasible and available to evaluate intravenous nutrient manipulations designed to limit body compositional loss following combat injury.

#### RECOMMENDATIONS

It is recommended that this model be used to collect data referable to the effect of altered nonprotein calorie/nitrogen ratios of intravenous solutions on body composition.

#### PUBLICATIONS

None

STUDY NO.	3	Effect of surgical dressings and procedures on skin bacteria surrounding venous catheters
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#### PROBLEM

In a recent report, the Center for Disease Control has suggested that the incidence of infectious complications from central venous catheters is directly related to the type of skin preparation prior to catheter insertion, the type of catheter care following insertion, and the duration of central venous catheterization. An additional factor is the occurrence of simultaneous sepsis from other sources in the patient (i.e., a patient with wound sepsis and concomitant bacteremia will have a higher incidence of catheter sepsis due to the foreign body of the catheter in this patient's blood stream).

The Department of Surgery, LAIR, has proposed guidelines for the optimal care of central venous catheters. The rationale for some of the procedures stems from empirical knowledge, extrapolation of in vitro

studies, and an old body of published in vivo investigations. Their particular procedures have not been prospectively examined from a microbial and ecologic viewpoint. To date, the experience reveals a 0% incidence of central venous catheter induced sepsis in over 3,500 patient days. Although the guidelines are probably responsible in part for the low incidence of nosocomial infections on the wards, supportive experimental data are lacking.

The Vietnam conflict saw the first wide-spread use of subclavian catheterization techniques for rapid fluid administration and monitoring of central venous pressures. The use of the subclavian vein as an easily accessible route for intravenous fluid administration and for central venous pressure monitoring was stressed by the fact that a detailed description of this technique was given in the NATO Handbook, Emergency War Surgery. In addition, Hardaway (NATO Handbook) reported that supraclavicular puncture of a subclavian vein was a routine method for administering intravenous fluids, obtaining venous blood samples, and measuring central venous pressure in the Vietnam conflict.

The combat injured soldier is at a particularly high risk for the development of a central venous catheter infectious complication for the following reasons: (1) skin preparation prior to catheter insertion is often necessarily minimal due to time constraints, and consequently, may be inadequate; (2) since no organized method of central venous catheter care has been established for the combat injured, it may be that care of the catheter entry site and care of the intravenous line to prevent infection is absent; and (3) the principal cause of postoperative death in combat injured individuals is sepsis. The septic origin is generally that of the combat wound. The presence of this uniformly infected wound in a patient with a chronically indwelling foreign body in his venous system significantly increases the changes of catheter colonization with resultant catheter-induced sepsis as well.

To highlight the concern over catheter sepsis under combat conditions, the CINCPAC 5th War Conference Manual has recommended: (1) changing central venous catheters every 48 to 72 hours, and (2) meticulous dressing changes, to include defatting the skin and application of antibiotic (Neosporin) ointment. This approach increases the risk of mechanical catheter complications (e.g., tension pneumothorax, hydro- or hemothorax, subclavian arterial injury, brachial plexus injury) due to repeated percutaneous subclavian catheterization. In addition, Neosporin ointment is not fungicidal, and in current practice of central venous catheter care, routine use of Betadine rather than Neosporin ointment is recommended. (There is recent evidence to suggest that the manner of skin cleansing is more important than the type of antibiotic ointment used in a civilian patient population.)

Therefore, studies to investigate a method of skin preparation that is effective and can be performed rapidly, in conjunction with studies to evaluate types of dressing techniques for maintenance of central



venous catheters should be extremely advantageous in preventing an accelerated incidence of catheter-induced sepsis in combat injured soldier.

The goals of this study are to optimize dressing care of central venous catheters inserted under combat conditions: (1) to improve or modify dressings and central venous catheter care and procedures which minimize risk of infection; and (2) to design a simple kit for combat conditions that will facilitate central venous catheter care.

#### RESULTS AND DISCUSSION OF RESULTS

Since this is a new protocol, no data have been generated to date. However, to allow this and other future clinical protocols to be implemented, a LAIR-based clinical service has been established at LAMC. To date this service has participated in the care of 140 patients totaling over 4,000 patient days of involvement. These are patients requiring intense intravenous nutritional support with nutrient demands and body compositional losses similar to those seen following combat injury.

#### CONCLUSIONS

A large patient data base has been established for clinical evaluation of the efficacy and safety of methods designed to supply intravenous nutrition to combat casualties. Data collection is highly organized with the use of computer technology and patient care is carefully supervised. This data base will prove invaluable for this and future clinical protocols.

#### RECOMMENDATIONS

It is recommended that new protocols be designed to: (1) predict those patients at risk from nutrient deficits following severe stress; (2) to monitor patient progress during intravenous nutrition when simple field-available laboratory techniques are used; and (3) to evaluate changes in nutrient supply on body composition and return to normal function.

#### PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>		2. DATE OF SUMMARY <sup>a</sup>		REPORT CONTROL SYMBOL		
				DA OE 6307		77 10 01		DD DR&E(AR)636		
3. DATE PREV. SUMMARY		4. KIND OF SUMMARY		5. SUMMARY SCTY <sup>a</sup>		6. WORK SECURITY <sup>a</sup>		7. REGRADING <sup>a</sup>		
77 07 19		H.Termination		U		U		NA		
								NL		
								8a. DMB'S INSTR'N <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		
								8b. SPECIFIC DATA - CONTRACTOR ACCESS <input type="checkbox"/> YES <input type="checkbox"/> NO		
								9. LEVEL OF SUM		
								A. WORK UNIT		
10. NO./CODES <sup>a</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY		62172A		3S162172A814		00		010		
b. <del>SPONSORING</del>		62772A		3S762772A814		00		010		
c. <del>SPONSORING</del>		CARDS 114 f								
11. TITLE (Precede with Security Classification Code) <sup>a</sup>										
(U) Systemic Factors in the Prevention of Wound Complications Following Combat Trauma										
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>										
003500 Clinical Medicine; 008800 Life Support; 016200 Stress Physiology										
13. START DATE			14. ESTIMATED COMPLETION DATE			15. FUNDING AGENCY			16. PERFORMANCE METHOD	
76 10			77 09			DA			C. In-House	
17. CONTRACT GRANT										
a. DATE/EFFECTIVE			EXPIRATION			18. RESOURCES ESTIMATE			a. PROFESSIONAL MAN YRS	
b. NUMBER <sup>a</sup> Not Applicable						PRECEDING				
c. TYPE			d. AMOUNT			FISCAL YEAR			b. FUNDS (in thousands)	
						77			1.0	
e. KIND OF AWARD			f. CUM. AMT.			CURRENT			34	
						78			0.0	
									00	
19. RESPONSIBLE DOD ORGANIZATION					20. PERFORMING ORGANIZATION					
NAME <sup>a</sup> Letterman Army Institute of Research					NAME <sup>a</sup> Letterman Army Institute of Research					
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					Department of Surgery					
					Presidio of San Francisco, CA 94129					
RESPONSIBLE INDIVIDUAL					PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)					
NAME <sup>a</sup> Canham, J.E., COL, MC					NAME <sup>a</sup> Gourlay, Stuart J., LTC, MC					
TELEPHONE <sup>a</sup> (415) 561-3600					TELEPHONE <sup>a</sup> (415) 561-3385					
21. GENERAL USE					SOCIAL SECURITY ACCOUNT NUMBER					
Foreign Intelligence Not Applicable					ASSOCIATE INVESTIGATORS					
					NAME <sup>a</sup> Caldwell, Michael D., MAJ, MC					
					NAME <sup>a</sup> POC:DA					
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>										
(U) Wound Healing; (U) Wound Infection; (U) Shock; (U) Trauma; (U) Nutrition; (U) Metabolism; (U) Acute Resuscitation										
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)										
<p>23. (U) Combat wounds are sustained in adverse environments and frequently they are wounds characterized by massive tissue devitalization produced by modern ordnance. Infection and delayed healing complicate these more frequently than wounds seen in civilian institutions. Wound complications resulting from minor as well as massive injuries not only jeopardize recovery but prolong hospitalization and delay return to duty. Roles played by systemic factors during wound healing are poorly understood. Under this work unit, the effects of various resuscitation modalities and post-traumatic metabolic regimens on wound healing and resistance to infection will be investigated. The results will be translated into management techniques to provide the optimal systemic milieu for combat wound healing.</p> <p>24. (U) Skin, subcutaneous, fascial, and gastrointestinal wounds will be created in rats, rabbits, and dogs subjected to hemorrhage, shock, and skeletal and soft-tissue trauma. The effects of various resuscitative regimens with blood and blood substitutes, and of post-traumatic metabolic support with micronutrients, hormones, vitamins, and trace elements on the healing and resistance to infection of these wounds will be evaluated with microbiological, biochemical, morphologic, and tensiometric techniques.</p> <p>25. (U) 76 10 - 77 09 A wound healing laboratory was established and methods were adapted to measure in soft tissue and osseous wounds the following: protein, collagen content, respiratory gases, and tensile and bursting strengths. Due to the departure of the principal investigator and a shortage of personnel, the work unit is being terminated. The established laboratory and techniques will be used to support osseous tissue healing investigations and surgical metabolism studies in other work units.</p>										

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 010 Systemic Factors in the Prevention of  
Wound Complications Following Combat  
Trauma

Under this work unit, laboratory methods were established for the measurement of hydroxyproline to estimate wound collagen content, serum and wound protein, blood and wound respiratory gases, and soft and osseous tissue bursting and tensile strengths. Biochemical standards and normal animal tissues were tested to perfect the methods, but no actual animal experiments were initiated due to departure of the principal investigator. Experimental methods developed under this work unit will be used to study osseous healing under another work unit.

## BODY OF REPORT

WORK UNIT NO. 010

Systemic Factors in the Prevention of  
Wound Complications Following Combat  
Trauma

### PROBLEM

Severe combat injury involves massive tissue destruction by high velocity projectiles. The inevitable contamination of such wounds by battlefield soil and enteric bacterial flora produces an inordinately high incidence of infectious wound complications. This contamination and the frequent concomitant occurrence of hypovolemic shock and systemic hypoxia combine to create a milieu favorable to delays in wound healing also. Wound infection and disruption due to delayed healing are much more frequent occurrences after combat injury than after civilian traumatic injury. These complications significantly prolong hospitalization and delay return to duty of the combat injured soldier.

The long range goals of this study are to investigate the ameliorating effects of battlefield resuscitation with blood, blood substitutes, and resuscitative solutions, and of nutritional and metabolic supportive regimens in the early postinjury period on the trauma and infection-mediated delays in wound healing and increased susceptibility to infection. Skin, subcutaneous, fascial, and gastrointestinal wounds in laboratory animals subjected to shock, hemorrhage, and soft and skeletal tissue trauma will be studied.

### RESULTS AND DISCUSSION OF RESULTS

A wound healing laboratory was established. An autoanalyzer technique for the measurement of hydroxyproline was developed and wet tissue processing techniques adapted to measure the collagen content of healing wounds. Blood gas analysis equipment was adapted to measure wound respiratory gases; serum and wound exudate protein measurement techniques were established for the spectrophotometer. An Instron tensiometer was adapted to measure the tensile strength of nonosseous tissues; compression testing accessories are being purchased to allow tensile strength testing in osseous tissues. Bowel and anastomotic strength measurements will be made by a simple manometric technique during insufflation with air. No actual animal experiments were performed under this work unit other than those on animal tissues from other work units to perfect test methods and a small pilot study involving 12 rats to test their tolerance to 6 different liquid diets.

The methods and equipment are available for wound healing studies and will be used mainly to study gastrointestinal healing and fracture healing under other work units.

CONCLUSIONS

None

RECOMMENDATIONS

None

PUBLICATIONS

None



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		3. REPORT CONTROL SYMBOL	
				DA OE 6105		77 10 01		DD DR&E (AR) 1036	
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12. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER			
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B. OPERATIONAL	62772A	3S762772A814		00		011			
C. OPERATIONAL	CARDS 114 t								
13. TITLE (Provide with Security Classification Code)									
(U) Problems in Abdominal Trauma									
14. SCIENTIFIC AND TECHNOLOGICAL AREA									
003500 Clinical Medicine; 012900 Physiology									
15. START DATE		16. ESTIMATED COMPLETION DATE		17. FUNDING AGENCY		18. PERFORMANCE METHOD			
76 01		78 06		DA		C. In-House			
19. CONTRACT GRANT				20. RESOURCE ESTIMATE		21. PROFESSIONAL MAN YRS		22. FUNDS (in thousands)	
A. DATES/EFFECTIVE:				PRECEDING					
B. NUMBER* Not Applicable				FISCAL YEAR		CURRENT		55	
C. TYPE				77		0.8			
D. KIND OF AWARD:				78		0.8		34	
23. RESPONSIBLE DOD ORGANIZATION				24. PERFORMING ORGANIZATION					
NAME* Letterman Army Institute of Research				NAME* Letterman Army Institute of Research					
ADDRESS* Presidio of San Francisco, CA 94129				ADDRESS* Div of Combat and Experimental Surg					
				Department of Surgery					
				Presidio of San Francisco, CA 94129					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. A-cadre; institution)					
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25. GENERAL USE				ASSOCIATE INVESTIGATORS					
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26. KEYWORDS (Provide with Security Classification Code)									
(U) Combat Injuries; (U) Stroma-Free Hemoglobin;									
(U) Abdominal Trauma; (U) Small Bowel Physiology; (U) Liver Morphology									
27. TECHNICAL OBJECTIVE* 28. APPROACH. 29. PROGRESS (Provide individual paragraphs identified by number. Provide last of each with Security Classification Code)									
<p>23. (U) Data from the Vietnam conflict indicate that the abdomen is the third most frequently wounded anatomical region. The high fatality rate (47.1%) of soldiers undergoing side-to-side small bowel anastomosis during WW II has been attributed to the massive amount of intestine resected in these cases. The objectives of these studies are (a) to develop a technique to replace the damaged small intestine with neosmall bowel mucosa derived from intact colonic segments; (b) to study intensively the short bowel syndrome by defining the morphological adaptive alterations in the small intestine after massive resection; and (c) to perfuse various abdominal organs with stroma-free hemoglobin solutions (SFHS) to determine the viability of organ function when these resuscitative fluids are used in the combat injured soldier.</p> <p>24. (U) Morphologic studies will define the hepatic and small bowel adaptive changes following massive resection using tissue from patients undergoing intestinal bypass procedures as experimental models. Animals with massive small bowel resections will be subjected to replacement of the resected bowel with mucosal stripped colonic wall to study the growth and absorptive capacity of neomucosa. The histologic and physiologic results of exchange transfusing animals with SFHS on abdominal organs will be studied.</p> <p>25. (U) 76 09 - 77 10 A study of the adaptive response of the liver and small intestine to intestinal bypass has been completed. The small bowel alterations increase the total functional absorptive surface area after surgery in a fashion similar to that observed after massive intestinal resections. Segments of mucosal denuded colon have been successfully transplanted into the small intestine after massive resections. Early growth of remaining small bowel mucosa onto the colonic surface has been documented. SFHS causes no adverse effects on the kidney and brain and appears to off-load oxygen to the liver. However, the rapid disappearance of SFHS from the intravascular space results in eventual hypovolemia and hepatic-ischemia.</p>									

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation

WORK UNIT NO. 011 Problems in Abdominal Trauma

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 The short bowel syndrome

STUDY NO. 2 The effects of stroma-free hemoglobin solutions on intra-abdominal organs

STUDY NO. 1. Large portions of small intestine may require resection following extensive gunshot and missile wounds. Since an insufficient functional absorptive area to transport nutrient material may remain, a considerable threat to life may result. As a part of this study, patients undergoing intestinal bypass procedures were used as a model to study small bowel and liver adaptation following massive loss of absorptive surface. The results of these studies indicated that with reduced absorptive surface area, there is increased deposition of lipid in the liver. This phenomenon is partially compensated for by intra-mitochondrial adaptive alterations in those cells near the portal triads, which are reversed after the period of maximum weight loss. The intestinal alterations result in an almost 300% increase in absorptive surface area in the functioning residual intestine secondary to an 80% increase in intestinal villus size. Ultrastructural studies revealed that all portions of the enlarged villi are capable of transporting nutrient material. A strong correlation existed between diminished weight loss and increased villus size.

As a potential solution to the problem of diminished intestinal absorptive surface area following missile injuries to the small bowel, a study has been undertaken to convert segments of unharmed colon into small intestine. Colonic segments (10 cm long) were denuded of mucosa and placed in continuity in the small bowel. Regrowth of the entire mucosal covering occurred in approximately 12 weeks. Growth occurred from both residual colonic cells and small bowel epithelium coming across the anastomoses. Further studies are aimed at eliminating all colonic epithelial regrowth, thus only small intestinal epithelium was allowed to recover the denuded surface.

STUDY NO. 2. The potential use of stroma-free hemoglobin solution (SFHS) as acute resuscitative fluids in the combat injured soldier has prompted further interest in their efficacy and toxicity. In order to evaluate the use of SFHS in key organs, rats were exchange transfused with either SFHS or other common resuscitative fluids (plasmanate, albumin, and lactated Ringer's solutions), and the morphological effects on liver, kidney, and brain were compared. The results indicated that during early time intervals after exchange transfusion, SFHS provides

protection from the changes of hypoxia seen in control animals. However, the short intravascular half-life of SFHS eventually results in hypovolemia and liver necrosis. There were no ultrastructural alterations observed in the brain, and no evidence of renal damage after SFHS administration.

## BODY OF REPORT

WORK UNIT NO. 011 Problems in Abdominal Trauma  
STUDY NO. 1 The short bowel syndrome

EX-1 Analysis of functional activity of neo-small-bowel mucosa  
in dogs

### PROBLEM

The complications of removing large portions of small intestine secondary to trauma, such as gunshot and missile wounds in the abdomen, pose considerable threat to patient life. The primary defect is an insufficient functional absorptive area to transport nutrient material. A potential solution involves increasing the absorptive surface area by converting a transposed segment of large intestine into small intestine.

### RESULTS AND DISCUSSION OF RESULTS

Four mongrel dogs underwent mobilization of 10 cm segment of proximal colon with its vascular supply intact. The colonic mucosa was removed by blunt dissection. An 80% small bowel resection was performed and the denuded colonic segment was placed in continuity with the residual small bowel. Normal colonic continuity was restored. At serial timed intervals (up to 6 months), biopsies were obtained from the colonic segments. A regrowth of mucosa covered the entire segment within 12 weeks. This neomucosa originated from both residual colonic cells and partially from adjacent small bowel epithelium. Three more dogs underwent the same procedure with the additional measure of treating the denuded colon with varying strengths of glutaraldehyde to remove the residual colonic epithelium. One-half percent glutaraldehyde seemed to remove the remaining colonic cells without damaging the entire bowel wall. The glutaraldehyde also provided a surface for growth of nascent small bowel mucosa.

### CONCLUSIONS

Mechanical stripping of the colonic mucosa and treatment with glutaraldehyde provide a surface on which small bowel epithelium will grow and possibly will repopulate the entire denuded colonic segment.

### RECOMMENDATIONS

Further studies are now in progress to evaluate the growth of small bowel epithelium onto the denuded colonic segment. Once this process is complete and the colonic surface is covered by this mucosa, the functional ability of the epithelium to transport nutrient material will be evaluated. Finally, animals which have undergone massive small bowel resections will receive a denuded colonic segment, and its



ability to improve the functioning absorptive surface will be analyzed to determine whether this approach will lessen morbidity following massive resections of small intestine necessitated by combat trauma.

#### PUBLICATIONS

None

EX-2 The intestinal bypass procedure for morbid obesity as an experimental model of acute intra-abdominal trauma requiring extensive small intestine resection

#### PROBLEM

To provide effective management for soldiers who have undergone massive small bowel resections following combat trauma requires a thorough understanding of the adaptive changes which occur in the remaining intestine and liver after the traumatized bowel is resected. Patients with intestinal bypasses to control obesity were selected as the model. Liver and intestinal alterations following the procedure were investigated. Attention was focused on several alterations which have been observed in experimental animals following massive intestinal resections.

#### RESULTS AND DISCUSSION OF RESULTS

Eleven patients underwent jejunoileal bypass for morbid obesity. Serial, peroral intestinal biopsies, and needle liver biopsies, were obtained prior to and at timed intervals following operation in both fasted and fat-fed states. Villus height increased asymptotically, reaching a plateau one year after operation with an increase of 80% in mean villus length. The pattern of post-bypass body weight reached a plateau at 63.9% of initial body weight and correlated linearly with villus height ( $r=0.97$ ) following an asymptotic curvilinear course. The time required to attain 90% of total body weight loss was 15.9 months. A study of intestinal fat absorption at the ultrastructural level showed that the enlarged villi are lined by functionally mature epithelium capable of transporting lipid. The total increment in absorptive surface area in the residual discontinuity small bowel was approximately 300%. It is suggested that these small intestinal alterations are adaptive in nature and enable some compensation for the loss of functioning absorptive surface area. Similar alterations are proposed for patients suffering from massive bowel resections secondary to trauma. Alterations in the liver after bypass included: (1) a temporary increase in steatosis which resolved in most patients with time; and (2) the formation of intramitochondrial filaments (IMFs) in periportal hepatocytes. The IMFs appeared to be related linearly to the amount of intracellular lipid ( $r=0.95$ ) and were postulated as providing a mechanism for the handling of increased quantities of hepatic lipid. As the steatosis improved with time after the bypass, the IMFs decreased in number or disappeared. The accumulation of

intracellular lipid caused a general crowding of intracytoplasmic organelles to the periphery of the hepatocyte. Although the relative density of the organelles (per  $\mu^2$  cytoplasm) markedly increased, there was no evidence of structural damage. This finding may explain the reversibility of hepatic steatosis without sequelae.

#### CONCLUSIONS

All patients undergoing the intestinal bypass procedure for obesity displayed fatty infiltration of the liver predominantly oriented around the central vein. Following bypass surgery, the degree of steatosis worsens and then gradually improves. Cellular lipid accumulation does not morphologically damage the intracytoplasmic organelles but, rather, concentrates them in the peripheral cytoplasm surrounding the lipid. Intramitochondrial filaments in the hepatocytes occur in association with fatty infiltration and are derived from mitochondrial cristae. The incidence of filaments in hepatocytes of control subjects is insignificant. The filaments most likely represent a response to an altered liver metabolic state. The alterations in the intestine are aimed at increasing the functional absorptive capacity of the small bowel after either massive resection or small bowel bypass.

#### RECOMMENDATIONS

None

#### PUBLICATIONS

1. FRIEDMAN, H.I., T.G. CHANDLER, and T.G. NEMETH. Hepatic intra-mitochondrial filaments in obese patients before and after the intestinal bypass procedure. *Gastroenterology* 73:1353, 1977
2. FRIEDMAN, H.I., T.G. CHANDLER, C.C. PECK, T.J. NEMETH, and S.K. ODUM. Adaptational changes in intestinal morphology and fat absorption following the intestinal bypass procedure for morbid obesity. *Surg, Gynecol, & Obstet*, in press

STUDY NO. 2

The effects of stroma-free hemoglobin solutions on intra-abdominal organs

#### PROBLEM

The development of a stroma-free hemoglobin solution (SFHS) which could transport oxygen and be used as a field resuscitative fluid would provide a considerable improvement in logistical support of the combat injured soldier. In order to provide additional information concerning the efficacy and toxicity of such a solution, the morphological effects of SFHS were studied on various organs including the liver, kidney, and brain.

## RESULTS AND DISCUSSION OF RESULTS

Rats were exchange transfused with either SFHS or other commonly employed resuscitative fluids (lactated Ringer's, plasmanate, or albumin) and the effects on liver, kidney, and brain at various time intervals after exchange were determined. At early time periods after exchange transfusion, SFHS appears to prevent the alterations of hypoxia observed with the other solutions. However, at later periods of time, as the SFHS is cleared from the intravascular compartment, the animals' blood volume decreases (42% at 6 hours). This finding may reflect an osmotic diuresis caused by SFHS crossing the glomerular basement membrane. Consequently, there is evidence of liver ischemia and necrosis at 12 and 24 hours after exchange transfusion that is not observed in control animals. SFHS is observed in the proximal and distal renal tubules. Hemoglobin also appears to be absorbed partially in proximal tubular cells. By 24 hours after exchange transfusion, most of the SFHS has left the kidney. Some absorption droplets are still seen in proximal tubular cells and a few distal tubules appear to contain hemoglobin. At the ultrastructural level, there is no apparent damage to the kidney tubules or glomerulus. The brain shows no adverse morphological effect from the SFHS. Two months after exchange transfusion all organs continue to appear normal.

## CONCLUSIONS

SFHS provides protection from the effects of hypoxia seen with other common resuscitative fluids at early time periods after administration. However, its short intravascular life span results in eventual severe hypovolemia and its sequelae. There is no adverse effect of SFHS on renal or brain ultrastructure.

## RECOMMENDATIONS

Additional experiments are being planned to see if booster infusions of SFHS can prevent the hypovolemia and liver necrosis. Other studies will focus on the potential problems of reduced  $P_{50}$ , reticuloendothelial blockade secondary to SFHS, enhancing the intravascular half-life of SFHS, and the problem of hepatitis antigens in SFHS.

## PUBLICATIONS

1. FRIEDMAN, H.I., F. DEVENUTO, T.F. ZUCK, P. MELLICK, and L. LOLLINI. The histological and ultrastructural effects of stroma-free hemoglobin solutions on liver, kidney, and brain. *Surgical Forum* 28:345, 1977
2. ZUCK, T.F., F. DEVENUTO, J.R. NEVILLE, and H.I. FRIEDMAN. Oncotic and oxygen transport effects of hemoglobin solutions. *In: Blood Substitutes*, edited by G.A. Jameson and T.J. Greenwalt. Alan R. Liss, Inc., New York (in press)



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DISSEM INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
76 10 01	D. Change	U	U	NA	NL		
10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62172A	3S162172A814		00	012		
b. <del>SECONDARY</del>	62772A	3S762772A814		00	012		
c. <del>THIRDARY</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Studies in Pulsatile Extracorporeal Circulation							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
008800 Life Support; 016200 Stress Physiology; 009800 Medical and Hospital Equipment							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 11		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: Not Applicable				FISCAL YEAR		88	
c. TYPE:				CURRENT			
d. KIND OF AWARD:				78		2.0	
e. CUM. AMT.						73	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				NAME: Rodkey, William G., CPT, VC POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup> (U)Pulse Pressure;(U)Combat Surgery;(U)Trauma;(U)Wet Lung Syndrome;(U)Pulsatile Perfusion;(U)Left Ventricular Function;(U)Oxyhemoglobin Dissoc							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective is to develop a subprimate model which is appropriate for total extracorporeal circulation and will permit precise measurements of ventricular hemodynamic and metabolic function. This model has been used to investigate the importance of pulsatile flow, and will be used to conduct perfusions with blood having differing oxyhemoglobin dissociation characteristics. Additional investigations will evaluate the effectiveness of acute resuscitation fluids on heart performance.</p> <p>24. (U) An extracorporeal circulation system capable of supplying whole body perfusions with pulsatile flow at various pulse pressures will be used to study left ventricular function and to determine the effects of perfusion made on myocardial flow distribution characteristics.</p> <p>25. (U) 76 10 - 77 09 A cardiovascular investigational laboratory is functioning for accurate measurements of stroke volume, dp/dt, ejection fraction, myocardial metabolism, and coronary flow distribution. Three studies in swine have been completed and demonstrated a beneficial effect of pulsatile perfusion during prolonged periods of ventricular fibrillation, but no effect if the heart is allowed to beat. Initial investigations using blood with different oxyhemoglobin dissociation characteristics revealed little effect of P<sub>50</sub> on left ventricular performance.</p>							



# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation  
WORK UNIT NO. 012 Studies in Pulsatile Extracorporeal Circulation

The following investigations are being conducted under this Work Unit:

STUDY NO. 1 The effect of variation in pulse pressure on left ventricular function in the dog and swine

STUDY NO. 2 The effect of variation in the oxyhemoglobindissociation curve on left ventricular function in swine

STUDY NO. 1. The purpose of this study is to test the hypothesis that pulsatile perfusion in an extracorporeal life support system is superior to continuous perfusion in preserving myocardial function. The preparation permits direct control of heart rate, stroke volume, and afterload for controlled measurement of left ventricular function. Myocardial metabolism and coronary flow distribution can be measured with arterial coronary sinus blood sampling and injection of radioactive labelled microspheres. Experiments in three groups of swine showed no effect of pulsatile perfusion on left ventricular function during short periods of bypass, regardless of whether or not the heart was beating, fibrillating, or working against a pressure load. Pulsatile perfusion did preserve left ventricular function more adequately than continuous perfusion when the heart was allowed to fibrillate over a two hour period.

STUDY NO. 2. The relationship between preservation of myocardial performance and the oxyhemoglobin dissociation curve of priming solutions is being investigated in the same swine isolated heart preparation. These studies are designed to determine whether or not the  $P_{50}$  of resuscitation fluids, including whole blood, is a significant determinant of recovery from hemorrhagic shock secondary to massive combat wounds. Preliminary experiments have indicated that variation in the  $P_{50}$  value does not have an effect on left ventricular function at normal arterial oxygen tensions and hemoglobin concentrations. Attempts are being made through further work, now in progress, to determine whether or not the oxyhemoglobin dissociation curve is important in preserving heart performance during combat injury situations involving hypoxia, hypovolemia, and/or anemia.

## BODY OF REPORT

WORK UNIT NO.	012	Studies in Pulsatile Extracorporeal Circulation
STUDY NO.	1	The effect of variation in pulse pressure on left ventricular function in the dog and swine

### PROBLEM

In designing an extracorporeal circulation system for prolonged life support in the injured combat soldier, there is controversy about the benefit or necessity of pulsatile flow. Evidence for the direct effect of pulse pressure on left ventricular function in such a system has not previously been available. Because of the technical complexity of pulsatile perfusion, continuous perfusion would be preferred in field settings. We have investigated the effects of pulsatile perfusion on the preservation of left ventricular function and have attempted to discern its importance in acute life support systems.

### RESULTS AND DISCUSSION OF RESULTS

Preliminary experiments established the animal model preparation, tested the pulsatile perfusion system, and confirmed the validity of left ventricular function measurements in both dogs and pigs. The initial experiment involving 12 dogs has been completed; it was demonstrated that pulsatile perfusion offered little advantage in preserving function in the beating heart in the dog.

To determine fully the importance of pulsatile flow in preserving left ventricular integrity, three groups of experiments were completed with the use of swine. Swine were chosen in the hope that between animal variation, as seen in the canine model, could be minimized. Also, swine were considered more appropriate because their physiology, when compared with the physiology of the dog, resembles more closely man's physiology, specifically in terms of cardiac output, cardiovascular reserve, ventricular performance, and coronary anatomy.

The three groups of experiments asked the following questions: (1) Is pulsatile perfusion important in the pressure loaded left ventricle? (2) Are coronary flow distribution and myocardial oxygen consumption changes found during fibrillation affected by pulsatile perfusion? (3) During longer periods of cardiopulmonary bypass, will pulsatile perfusion protect against the deterioration in left ventricular function and endocardial flow noted with ventricular fibrillation? All 3 groups of experiments have been completed, and the results are either published or in press. The first question has been answered in the negative, and we failed to find any effect of pulsatile perfusion in the beating empty or pressure loaded heart. We also failed to show

any benefit of pulsatile perfusion during short periods of cardiopulmonary bypass in the fibrillating heart. Rhythm changes corresponded with changes in the endoepicardial flow ratio, but the perfusion modality (pulsatile or continuous) had little effect. Myocardial oxygen consumption, total coronary flow, and lactate extraction were not affected by the perfusion modality. The third group of experiments has substantiated a beneficial effect of pulsatile perfusion. If the fibrillating heart is supported for a 2-hour period on cardiopulmonary bypass with continuous flow, we found a decrease in left ventricular function of approximately 25% and a similar reduction in the endoepicardial flow ratio. If fibrillating hearts were supported with pulsatile cardiopulmonary bypass, these changes in left ventricular function and flow distributions fail to occur.

#### CONCLUSIONS

This study has demonstrated, in two different experimental animals, that compared with continuous perfusion, pulsatile extracorporeal perfusion has little to offer in terms of preserving myocardial performance and coronary flow in the beating heart. During prolonged support of the fibrillating myocardium, pulsatile perfusion may have a place in preserving left ventricular function.

#### RECOMMENDATIONS

The importance of pulsatile perfusion in a total life support system for the combat injured soldier is probably limited to maintaining the integrity of critical organs other than the heart. Since the benefits of pulsatile perfusion for the heart are limited, further work in pulsatile perfusion should be directed towards studying the effects on other critical organs (e.g., kidney, brain, and liver).

#### PUBLICATIONS

1. STOWE, D.F., J.V. TYBERG, D.G. MATHEY, W.Y. MOORES, R.M. TOWNSEND, P. KABRA, K. CHATTERJEE, and W.W. PARMLEY. Regional myocardial ischemia in the pig: A model for mechanics and metabolism. (Abstract) *The Physiologist* 19:381, 1976
2. TYBERG, J.V., D.G. MATHEY, D.F. STOWE, W.Y. MOORES, R.M. TOWNSEND, P. KABRA, K. CHATTERJEE, and W.W. PARMLEY. Tight coupling between segment stroke work, coronary blood flow, and energy metabolism during graded myocardial ischemia. (Abstract) *Clin Res* 25:95A, 1977
3. MOORES, W.Y., J.P. HANNON, J.D. CRUM, D. WILLFORD, W. RODKEY, and J. GEASLING. Coronary flow distribution and dynamics during continuous and pulsatile extracorporeal circulation in swine. *Ann Thorac Surg*, 1977 (in press)

4. MOORES, W.Y., J.P. HANNON, J.D. CRUM, D.C. WILLFORD, W.G. RODKEY, and J.W. GEASLING. Coronary flow distribution during continuous and pulsatile extracorporeal perfusion in the pressure loaded and unloaded swine left ventricle. (Abstract) Fed Proc 36:599, 1977
5. MOORES, W.Y., O. GAGO, J.D. MORRIS, and C.C. PECK. Serum and urinary amylase levels following pulsatile and continuous cardiopulmonary bypass. J Thorac Cardiovasc Surg 74:73, 1977
6. MOORES, W.Y., J.P. HANNON, J.D. CRUM, and D.C. WILLFORD. The continuous and pulsatile extracorporeal coronary perfusion in the beating and fibrillating swine myocardium: Effects on left ventricular function. Surg Forum, 1977
7. GLANTZ, S.A., G.A. MISBACH, W.Y. MOORES, D. MATHEY, J. LEKVEN, D. STOWE, W.W. PARMLEY, and J.V. TYBERG. The pericardium substantially affects the dog ventricular diastolic pressure relationship. (Abstract) Circulation (in press)
8. TYBERG, J.V., G.A. MISBACH, S.A. GLANTZ, W.Y. MOORES, and W.W. PARMLEY. A mechanism for shifts in the diastolic left ventricular pressure volume curve: The role of pericardium. European J Cardiol (in press)

STUDY NO. 2

The effect of variation in the oxy-hemoglobin dissociation curve on left ventricular function in the swine

#### PROBLEM

Recently, with the understanding that the oxyhemoglobin dissociation curve is affected by concentrations of 2,3-DPG, and that stored blood has a low 2,3-DPG level, there is concern that massive transfusions with blood having a long shelf life may have a detrimental effect on oxygen delivery to critical tissues. Myocardial function is intimately tied to adequate oxygen transport, and in the combat injured soldier, depressed heart performance may result if oxygen transport is not optimal. Some inadequately controlled studies have suggested that there is a relationship between  $P_{50}$  and left ventricular performance. If an adequate  $P_{50}$  is crucial in preserving heart performance during periods of combat injury, then aged blood with a low  $P_{50}$  and low 2,3-DPG may have limited usefulness, and fresh blood or blood with enriched 2,3-DPG may have to be available. If  $P_{50}$  is not a major determinant of left ventricular function, then greater use of blood with longer shelf ages may be employed, especially during combat situations that would require massive transfusions and optimum utilization of blood bank resources.



## RESULTS AND DISCUSSION OF RESULTS

Our in situ perfused swine heart model is being utilized for this study. Left ventricular function and metabolic responses are being evaluated and, in addition, myocardial tissue  $PO_2$  and  $PCO_2$  tensions are being directly measured with a medical mass spectrometer.

This study is in a preliminary stage, but we have established an in vitro capability for enriching or depleting 2,3-DPG and subsequently altering the oxyhemoglobin dissociation curve and the measured  $P_{50}$ . The technique of performing an exchange transfusion on cardiopulmonary bypass has been well established. Our initial results show that with a normal hematocrit and arterial oxygen tension  $P_{50}$  changes in the range of 24-50 torr do not affect heart performance. We found that myocardial oxygen extraction was higher with the higher  $P_{50}$ , however.

## CONCLUSIONS

If our initial results are substantiated, we can conclude that under normal hemodynamic conditions,  $P_{50}$  is not an important determinant of left ventricular function. The crucial question of the role of oxyhemoglobin dissociation curve changes in abnormal hemodynamic situations (hypoxia, hypotension, anemia), as would be encountered in a combat injury situation, is being addressed with experiments evaluating these conditions.

## RECOMMENDATIONS

The question of the role of  $P_{50}$  changes in blood and oxygen transporting resuscitation fluids is one that is crucial to the optimum resuscitation of soldiers injured in combat. Our initial results seem to indicate that, if adequate arterial pressure and oxygen tension are maintained,  $P_{50}$  is not a factor in determining left ventricular function, and blood with a low 2,3-DPG concentration may be adequate, even if massive transfusions are needed. Experiments defining the role of the oxyhemoglobin curve on left ventricular function in a massively injured soldier with complicating factors of hypoxia, hypotension, and/or anemia should receive a high priority in this study.

## PUBLICATIONS

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL: DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DDB'S INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62172A	3S162172A814	00	013			
b. <del>SECONDARY</del>	62772A	3S762772A814	00	013			
c. <del>THIRDARY</del>	CARDS 114 f						
11. TITLE (Precede with Security Classification Code)*							
(U) Effect of Blood-Oxygen Affinity During Experimental Hemorrhagic Shock and Hypoxemia							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 012900 Physiology; 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: Not Applicable				FISCAL YEAR		77	
c. TYPE:				CURRENT		0.5	
d. AMOUNT:				78		0.5	
e. KIND OF AWARD:				43			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				POC: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Resuscitation Solutions;							
(U) Experimental Hemorrhagic Shock; (U) Trauma; (U) Blood-gas Transport							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The objective is to evaluate the oxygen transport function of blood and resuscitation solutions, particularly with reference to the role of hemoglobin-oxygen affinity in modifying physiologic responses of military personnel to trauma and environmental stress.							
24. (U) The approach to this problem incorporates three areas of effort: (a) design and improvement of techniques and equipment for performing unusual or difficult biomedical measurements related to oxygen transport function, (b) theoretical analysis of the oxygen transport function of blood and related physiologic systems, and (c) use of experimental animals, particularly specialized animal preparations such as the swine heart by-pass model (see LAIR work unit 012), to test and evaluate relevant questions regarding the implications of hemoglobin-oxygen affinity to tissue oxygen transport in the combat wounded soldier.							
25. (U) 76 10 - 77 09 Further observations have been carried out in rats by using various agents to manipulate oxygen affinity and study the systemic consequences. Cyanate-treated rats had hepatic lesions demonstrable with electron microscopy. It is not clear whether these lesions arise by direct action of cyanate or indirectly from the cyanate-induced leftward shift of the hemoglobin-oxygen affinity curve. Theoretical and experimental studies of the oxygen transport properties of stroma-free hemoglobin indicate that the minimum effective replacement volume of this material is about 75% of the normal circulating blood volume. Several collaborative efforts have been established (see LAIR work units 075, 003, and 012) to pursue questions associated with hemoglobin-oxygen affinity change and resuscitation from trauma.							

# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation

WORK UNIT NO. 013 Effect of Blood-Oxygen Affinity During  
Experimental Hemorrhagic Shock and  
Hypoxemia

Rats given water ad libitum with added sodium cyanate develop abnormal (by electron microscopy) mitochondria in liver tissue as well as increased hemoglobin-oxygen affinity. Whether these lesions are produced directly by carbamylation or indirectly through the effect of increased blood-oxygen affinity is not clear. The effect of various ratios of stroma-free hemoglobin solution (SFHS) and whole blood on the  $P_{50}$  and heme-heme interaction of these mixtures was experimentally determined. With the use of these figures, it was estimated that, compared to asanguineous replacement, no net benefit to oxygen transport can be expected by using SFHS (7.5 grams percent hemoglobin) below a 75% blood volume replacement level. Both in vivo aging and in vitro storage of erythrocytes modify the heme-heme interaction observed in whole blood. The full effect of changes in oxygen transport when stored blood is used is not reflected by measurements of  $P_{50}$  alone. Decreases in  $P_{50}$  and heme-heme interaction, such as those observed in many ICU patients, can grossly degrade oxygen transport function, particularly when accompanied by anemia and/or carbon monoxide inhalation during smoking.



## BODY OF REPORT

WORK UNIT NO. 013

Effect of Blood-Oxygen Affinity During  
Experimental Hemorrhage Shock and  
Hypoxemia

### PROBLEM

A continuous supply of oxygen is a major requirement for maintenance of vital functions in man and animals. Compared to food and/or water deprivation, which may be tolerated in man for prolonged periods, oxygen deprivation can be fatal in minutes. Although some tissues withstand the effects of oxygen deprivation better than others, the body as a whole tolerates a deficit oxygen economy only to the extent that it can activate a variety of compensatory mechanisms designed to sustain the minimum metabolic needs of the tissues during emergencies. Trauma associated with bleeding produces an immediate threat to the oxygen economy of the body, and the effectiveness of compensatory adjustments designed to cope with this situation is a decisive factor in the clinical consequences of such trauma. Apart from the potentially lethal effects of oxygen deprivation, tissue hypoxic episodes can lead to permanent functional damage and prolong recovery from the basic injury.

The occurrence of trauma and hemorrhage in a remote and hostile battlefield setting presents several unique problems not ordinarily encountered. Loss of consciousness, for instance, is sometimes considered to be beneficial after trauma because it tends to place the body in a horizontal position favoring cardiac output and maintenance of circulation to the brain. Such a response may be hazardous even under normal circumstances, but its occurrence under battle conditions definitely jeopardizes the individual's already precarious safety and his potential for self-preservation. Medical assistance, furthermore, may be delayed by the exigencies of warfare, thus extending the stress upon compensatory reserves. Finally, blood replacement therapy, once available, may be compromised by the quality of blood products available in forward areas. Either separately or in combination, these factors make it significantly more difficult for the military physician to normalize the oxygen economy prior to permanent functional damage or in time to prevent prolonged recovery times from the basic injury.

Thus, there are unique circumstances justifying the military need to understand the complex compensatory mechanisms that sustain the oxygen economy of the body. The objective of this work unit is to evaluate the oxygen transport function of blood and resuscitative solutions, particularly with reference to the role of hemoglobin-oxygen affinity in modifying the compensatory reserves of combat personnel to trauma and environmental stress.



## RESULTS AND DISCUSSION OF RESULTS

Treating rats with sodium cyanate added to drinking water has been found to cause abnormal morphologic changes in the liver, as well as a leftward shift in the oxyhemoglobin dissociation curve. The hepatic lesion observed ultrastructurally is similar to that seen with the use of stroma-free hemoglobin solutions (SFHS), and involves the mitochondria of those cells nearest the central vein. Vacuolization is also observed and is most prominent in those cells nearest the central vein. These changes are suggestive of those seen with hypoxia; it is for this reason that the lesion is considered to be the result of the concurrent shift in the oxygen affinity curve rather than direct carbamylation of cell proteins by cyanate. Presumably, direct carbamylation would have exercised its effect in a more general pattern. The central hepatic vein appears to be particularly susceptible to hypoxia because the circulation pattern in the liver presents the cells surrounding this area with blood of extremely low  $O_2$  content. The experimental use of cyanate to treat sickle cell disease has not been reported to cause any type of cellular lesion. Previous findings stemming from this work unit have emphasized that intensive care patients, particularly heart disease patients, have a marked tendency to display leftward shifts in the hemoglobin-oxygen affinity curve. The more definitive association of cellular pathology with an altered oxygen transport function, as represented by the present observations in cyanate-treated rats, offers the prospect of an improved experimental approach to this problem. Many previous experimental investigations of this problem have been concerned with acute effects, and have involved relatively short periods of experimental observation. The present results and those reported in ICU patients imply that the effects of altered hemoglobin-oxygen affinity may be cumulative and slow acting, particularly where alternative physiologic responses are capable of providing short-term compensatory balance to an altered oxygen economy.

Experimental measurements made from mixtures of SFHS and whole blood have shown that as the percentage of SFHS increases, the  $P_{50}$  of these mixtures decreases in an approximately linear fashion. In the absence of SFHS, normal whole blood has a  $P_{50}$  of about 27 mm Hg; 100% SFHS usually has a  $P_{50}$  of about 12-15 mm Hg. A 50% mixture has a  $P_{50}$  of about 20 mm Hg. The apparent heme-heme interaction of these mixtures also varies (the minimum value is about 2.0 to 2.2 with 50% SFHS). Aging of whole blood erythrocytes has been found to cause a similar change in both  $P_{50}$  and the heme-heme interaction:  $P_{50}$  decreases linearly with age (and loss of 2,3-DPG) from a value of about 27 mm Hg to 12 to 15 mm Hg; heme-heme interaction diminishes to a minimum value of 2.0 to 2.2 when 2,3-DPG is depleted to about one-half its normal value. Subsequent losses in 2,3-DPG in aging cells, as well as increases in the percentage of SFHS above the 50% level, result in increases in the heme-heme interaction. By using the Hill equation and several assumptions regarding tissue oxygen tension, it has been possible to compute the tissue oxygen transport effectiveness of SFHS at various

replacement volumes. These calculations indicate that, compared to asanguineous transfusions, it is necessary to replace at least 75% of the circulating blood volume with 7.5 grams percent SFHS before the tissues will realize any net improvement in oxygen unloading. This result is due primarily to the extreme leftward position of the  $P_{50}$  and the low cooperativity found in SFHS-whole blood mixtures. Apparently, SFHS would not be clinically effective at low replacement volumes; the material, in fact, might be counterproductive compared to asanguineous fluids at replacement levels of less than 75%. This result emphasizes the need to improve the oxygen delivery characteristics of SFHS if it is to gain general clinical applicability.

Further analysis of the hemoglobin-oxygen affinity characteristics of ICU patients has shown that, in addition to a frequently extreme leftward shift in the curve, many of these patients simultaneously have anemia. The extent of the anemia and alteration in the oxygen affinity characteristics of hemoglobin would be clearly handicapping in terms of maintaining a normal oxygen economy. Moreover, many of these patients present histories of excessive tobacco consumption that is known to be associated with high levels of CO-hemoglobin. The oxygen transport function of an individual who is already handicapped by an increased hemoglobin-oxygen affinity and anemia will become severely limited by the levels of CO-hemoglobin that are attained in heavy smokers. In some cases, in fact, the tissue oxygen delivery would be reduced to as little as 30% of normal. In an organ such as the heart, that relies heavily on autoregulation to maintain tissue oxygenation, the co-existence of coronary insufficiency and limited oxygen delivery capacity of hemoglobin would be expected to affect the organ's oxygen economy to a clinically significant extent.

#### CONCLUSIONS

Increased hemoglobin-oxygen affinity can lead to hepatic lesions similar to those found in hypoxia. The effects of hemoglobin-oxygen affinity change may be manifested slowly rather than acutely; many experiments designed to test the effects of altered hemoglobin-oxygen affinity have not accounted for this possibility. The heme-heme interaction of stored blood and aging erythrocytes can be greatly modified from normal. This change can affect oxygen delivery to tissues in a significant manner, a factor that few investigators have considered. At least a 75% replacement with SFHS is required before any net advantage to oxygen transport is obtained with this material compared to asanguineous replacement. Changes in hemoglobin-oxygen affinity may play a contributory etiologic role in some diseases, particularly heart diseases, and be a factor in the recovery of patients who have experienced a deficit oxygen economy.

### RECOMMENDATIONS

The possible relationship between an altered hemoglobin-oxygen affinity and tissue morphologic changes should be studied in more detail, including tissues other than the liver and species other than the rat.

Attempts should be made to improve the oxygen affinity characteristics of SFHS. The possibility of using bovine hemoglobin, which produces a SFHS with a  $P_{50}$  of about 28 mm Hg, should be investigated.

The potential long term effects of altered hemoglobin-oxygen affinity needs to be emphasized and experimentally investigated, since such effects may modify both the response to and subsequent recovery from trauma.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DISSEM INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
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10. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62172A	3S162172A814		00		014	
b. OTHER WORKING	62772A	3S762772A814		00		014	
c. OTHER WORKING	CARDS 114 f						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Care of the Combat-Injured Eye							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 Clinical Medicine; 012900 Physiology							
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17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER <sup>a</sup> Not Applicable				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE:				CURRENT		32	
d. KIND OF AWARD:				78		0.2	
e. AMOUNT:				78		32	
f. CUM. AMT.				78		32	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129				ADDRESS <sup>a</sup> Presidio of San Francisco, CA 94129			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Applicable				ASSOCIATE INVESTIGATORS			
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				NAME:			
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23. KEYWORDS (Precede EACH with Security Classification Code) (U) Ocular Trauma; (U) Vitrectomy; (U) Dark Adaptometer; (U) Traumatic Cataract; (U) Traumatic Vitreopathy; (U) Ocular Electrophysiology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Injuries involving penetration of the eye often have an unfavorable outcome because of disruption of the normal anatomy of the eye by fibrocellular sheets and bands of tissue which develop during the healing process. This disruptive process may be modified by early aggressive surgery or drug therapy. The Army has no screening instrument to measure dark adaptation of the eye. Such an instrument is needed to screen ground troops and aviation personnel, and to monitor laser workers.</p> <p>24. (U) Massive ascorbate doses are given to rabbits after microsurgical repair of standardized ocular trauma. Aqueous electrolyte, ascorbate and biochemical parameters are followed; electrophysiologic and anatomic studies are performed to assess effect on ocular wound healing. Changing the duty cycle of light-emitting diodes by micro-computer and coupling with recording systems allows screening dark adaptometry to be performed with minimal technician time.</p> <p>25. (U) 76 10 - 77 09 The standardized ocular wound model and repair are developed and an ocular electrophysiology laboratory has been established. The ascorbate study is underway. The prototype, two-color, light-emitting diode dark adaptometer is developed and human use approval has been granted. Normative curves are being established. A field unit is being constructed.</p>							



# ABSTRACT

PROJECT NO. 3S762772A814 Military Trauma and Resuscitation

WORK UNIT NO. 014 Care of the Combat-Injured Eye

The following investigations have been conducted under this Work Unit:

STUDY NO. 1 Effects of early vitrectomy on induced penetrating ocular injuries

STUDY NO. 2 Dark adaptometer prototype

STUDY NO. 1. An animal model was established for the study of the effects of early vitrectomy on the outcome of severe penetrating ocular trauma. The healing of the rabbit eye was shown to be sufficiently different from that of the human eye so that this model could not be used. A recent clinical report on human ocular trauma indicates that vitrectomy is effective and should be performed as soon as possible if infection or reactive foreign bodies are suspected, and that vitrectomy should be delayed 5 to 10 days if these factors are not present. It is recommended that the military have a vitrectomy capability near the combat theater in order to salvage as many combat injured eyes as possible.

STUDY NO. 2. A prototype screening dark adaptometer has been developed. This device allows dark vision testing without the labor commitment of technicians required by other systems of dark vision testing. The laboratory prototype is undergoing normative testing. A field device is being developed for possible evaluation in screening combat troops and aviation personnel and for evaluating the ocular effects of chronic exposure to low level coherent light sources.

## BODY OF REPORT

WORK UNIT NO.	014	Care of the Combat-Injured Eye
STUDY NO.	1	Effect of early vitrectomy on induced penetrating ocular injuries

### PROBLEM

The incidence of penetrating ocular injuries during wartime has steadily increased. During both World War I and World War II, ocular injuries represented approximately 2½% of all casualties, whereas in the Korean Conflict the percentage rose to 8% and in the Israeli-Arab War and Vietnam Conflict the figures are 10% and 15%, respectively. Over 30% of the ocular injuries are penetrating wounds with their attendant sequelae of blindness. During the past 6 years, new instruments have been developed which allow the ophthalmic surgeon to remove opaque and damaged vitreous and lens and to sever or remove intraocular fibrous strands which cause traction detachments of the retina. By removing opacified vitreous and lens tissue, the surgeon is able to visualize the interior of the eye so that retinal holes may be repaired, foreign bodies removed, and the stimulus minimized for intraocular inflammation and fibrous proliferation. The broad objective of this investigation is to determine when an extensive surgical debridement of damaged vitreous and lens is most beneficial following a standardized penetrating trauma of the eye.

### RESULTS AND DISCUSSION OF RESULTS

A system of electroretinography was established for the experimental rabbits and a pneumatically-operated device constructed for delivering standardized penetrating trauma to the eye. A series of 13 rabbits underwent a single entry, standardized ocular trauma and repair; these animals were followed to study the sequelae of injury in this model. The results indicate that rabbit eyes respond differently than would be expected for human eyes which were similarly traumatized. The rabbit eyes developed neither complete lenticular opacification nor fibrous proliferation occluding the optical axis, even though the injury included cornea, sclera, iris, ciliary body, lens, vitreous, and retina. Another series of rabbits received 2 ocular wounds to simulate the "double perforating" or through-and-through wounding of human eyes. Again, the gross and microscopic results indicate that this rabbit model for penetrating ocular trauma is not sufficiently analagous to human injury to justify its use in assessing the potential value of extensive intraocular debridement in human eyes which have received similar trauma.

In a recent analysis of 51 human cases of severe penetrating injury involving the posterior segment, vitrectomy was recommended immediately in instances where the clinician suspects intraocular infection

or a retained reactive intraocular foreign body; otherwise, immediate primary closure and vitrectomy 5 to 10 days later is recommended (B.P. Conway and R.G. Michels, Presentation, American Academy of Ophthalmology and Otolaryngology, Dallas, Texas, 7 October 1977).

#### CONCLUSIONS

The rabbit eye is not an appropriate model for studies related to the effect of vitrectomy on similarly traumatized human eyes.

#### RECOMMENDATIONS

On the basis of recent human clinical experience with vitrectomy, the U.S. Army Health Services Command should strongly consider having skilled ophthalmologists (with sufficient training in vitrectomy and retinal surgery) near enough to the combat theater to utilize this technique in the care of combat injured eyes. The necessary equipment should be available for immediate transport in the event that armed conflict should occur.

STUDY NO.      2                      Dark adaptometer prototype

#### PROBLEM

The U.S. Army has no efficient means of screening the night vision of combatants, even though intensive and sustained conflict may occur during nighttime. A screening dark adaptometer might also prove useful in assessing the effect of drugs, combat environmental pollutants, and stress on night vision, and in assessing changes of retinal function due to chronic exposure to low levels of coherent light.

#### RESULTS AND DISCUSSION OF RESULTS

A prototype dark adaptometer has been developed which utilizes light-emitting diodes (LEDs) as the stimulus source. The apparent intensity of emitted light is controlled by varying the duty cycle of the LEDs by a microcomputer. A real time data display is achieved on either a cathode ray tube or an X-Y recorder for permanent records. No technician time is required during the testing period since the test subject signals the computer when the threshold stimulus is seen; thus, one technician may test 6 subjects simultaneously. Both rod and cone function are tested during the same session by alternating deep-red LEDs (for cones) and green LEDs (for rod function).

Preliminary mathematical modeling of the data suggests that a single dark adaptation curve is described by

$$\log I = \log I_{\infty} + (\log I_0 - \log I_{\infty})e^{-\alpha t}$$

where  $I_0$  is the initial threshold of the test stimulus after a set period of pretest light adaptation, and  $I_{\infty}$  is the absolute threshold

of the individual after full dark adaptation has occurred. Use of this model allows statistical analysis of data.

Appropriate human use committee approval has been received, and a normative data base is being obtained from volunteers.

#### CONCLUSIONS

The results from the prototype device appear extremely promising, and a prototype field device is being developed.

#### RECOMMENDATIONS

The development and testing of a screening dark adaptometer for military use should be continued.

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None



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## APPENDIX B

### DIRECTORY OF OFFICERS AND SENIOR PROFESSIONAL STAFF

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	Mary Jo Romano, CPT, AN B.S. (Villanova Univ.)
	Jacqueline Stewart, GS07 B.S. (Univ. of Colorado)
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Department of Tropical Medicine Chief	Moufied A. Moussa, LTC, MS Ph.D. (Univ. of Illinois)
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Division of Combat Experimental  
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